NOVEL DUAL-ACTING NITROXIDE-BASED HYBRID ANTIOXIDANTS

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Dedicated To My Family

Abstract

Chronic inflammation and oxidative stress are two common biological processes known to play significant roles in the mechanisms underlying the pathogenesis of many human inflammatory and neurodegenerative diseases such as atherosclerosis, rheumatoid arthritis, Parkinson's disease, and some cancers. As a wider strategy towards developing new therapeutics for managing chronic inflammation and oxidative stress-related diseases/disorders, this project employed the pharmacophore hybridization strategy to design, synthesize and evaluate the therapeutic efficacy of novel potential hybrid nitroxide-based antioxidants that may regulate multiple biological targets simultaneously (dual-acting). The approach combines various nitroxide compounds with known pharmacophores - mainly the NSAID and sympathomimetic stimulants class of drugs. The hybrid agents were constructed by either merging/overlapping the two structural subunits or via cleavable (ester and amide bonds) and non-cleavable (amine bond) linkers.

Three novel, potential dual action nitroxide-salicylate hybrids were successfully synthesized by overlapping similar structural subunits of the parent molecules. The merged analogues displayed moderate antioxidant and anti-inflammatory effects by inhibiting the MPO-mediated hypochlorous acid production system and the cyclooxygenase (COX) enzymes respectively. Although the results are encouraging, notably, the COX inhibitory effects were only significant after longer incubation times.

In addition to the merged hybrid nitroxide-salicylates, thirty novel cleavable (ester and amide bond linkers) and non-cleavable nitroxide-NSAID conjugates (amino linker) were synthesized. The hybrid nitroxide-NSAID conjugates displayed significant antioxidant and anti-inflammatory effects on the A549 Non-Small Cell Lung Cancer cells. Notably, the ester-linked nitroxide conjugate **140** possessed better anti-inflammatory effects (inhibitory effects on the COX enzyme) than parent aspirin **136**, and showed a better ROS scavenging activity than the versatile antioxidant, TEMPOL **102**. In addition to the antioxidant and anti-inflammatory effects, the hybrid conjugates also displayed promising anticancer effects. The nitroxide-NSAID

conjugates were shown to inhibit the proliferation of A549 Non-Small Cell Lung Cancer cells. Clonogenic assay studies of A549 cells indicated that combining the nitroxide-NSAID conjugates with anticancer agents such as gefitinib produced a synergistic effect on inhibiting A549 cells growth.

Although the key and ultimate step (dirhodium-catalyzed intermolecular C-H insertion of methyl phenyldiazoacetate) in the synthesis of the Ritalin-nitroxide analogue was unsuccessful, the Schöllkopf's bis-lactim ether asymmetric synthesis of α-amino acids approach was successfully employed to synthesize the target L-Dopa nitroxide derivative. By combining the Schöllkopf's methodology with the palladium-catalyzed Sonogashira coupling reaction, a rigid alkyne-linked chiral α-amino acid site directed spin label (SDSL) was also synthesized. The alkyne-linked α-amino acid was designed for studying the structure and conformational dynamics of peptides and proteins, and for monitoring ROS-induced cellular oxidative stress in diseases such as PD by both EPR and fluorescence techniques.

The synthesis of a series of isoindoline-based (tetramethyl and tetraethyl-substituted) catecholamine and benzodioxole compounds and their nitroxide derivatives is also described. As the first class of catechol-based nitroxides, the target nitroxide-catecholinic-hybrids were mainly designed as potential dual-acting antioxidant-sympathomimetic agents which could also be readily employed as synthetic templates/precursors for building functional materials. Since the therapeutic benefits of common catechol and benzodioxole-based pharmacophores (such as dopamine and L-Dopa) have been limited by the oxidative stress-induced cellular damage, the hybrid catecholic-nitroxides could provide effective antioxidant intervention (in addition to their potential sympathomimetic effects). The hybrid catecholinic compounds are also designed as potential substrates for building new nanocomposite drug delivery systems, sensing and electronic devices, metal complexes, catalysis and for (bio)chemical reactions.

Publications From This Work

Papers

Thomas, K., Chalmers, B. A., Fairfull-Smith, K. E., & Bottle, S. E. (**2013**). Approaches to the Synthesis of a Water-Soluble Carboxy Nitroxide. *Eur J Org Chem*, *2013*(5), 853-857.

Moody, T. W., Jensen, R. T., Thomas, K., Fairfull-Smith, K. E., Bottle, S. E., Ridnour, L., & Wink, D. A. (**2015**). Nitroxide-aspirin conjugates: A new class of NSAIDs. *Cancer Research*, *75*(15 Supplement), 4539-4539.

Blinco, J. P., Bottle, S. E., Fairfull-Smith, K. E., Simpson, E., & Thomas, K. "Synthesis of Nitroxides and Alkoxyamines". *Nitroxide Mediated Polymerization: From Fundamentals to Applications in Materials Science*, 114.

Lectures

Komba Thomas, Kathryn E. Fairfull-Smith and Steven E. Bottle "Hybrid Nitroxide-Aspirin-Based Dual Pharmacophores" ARC Centre of Excellence for Free Radical Chemistry and Biotechnology Spring Carnival (Wollongong, November **2012**)

Komba Thomas, Kathryn E. Fairfull-Smith and Steven E. Bottle "Hybrid Nitroxide-NSAID Conjugates" Brisbane Biological and Organic Chemistry Symposium (Brisbane, December **2012**, Student First Prize)

Komba Thomas, Kathryn E. Fairfull-Smith and Steven E. Bottle "Hybrid Dual-Action Nitroxide-Based Antioxidants" Nanotechnology and Molecular Science HDR Symposium (QUT, February **2013**, Student First Prize)

Komba Thomas, Kathryn E. Fairfull-Smith and Steven E. Bottle "Dual-Action Pharmacophore Hybridization: Combining Antioxidant Nitroxide with L-Dopa as Potential Parkinson's Disease Therapy" Brisbane Biological and Organic Chemistry Symposium (Brisbane, December **2014**).

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List of Abbreviations

[M]	molecular ion
AA	arachidonic acid
AAD-2004	2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic acid
AADC	aromatic L-amino acid decarboxylase
Ac6c	1-aminocyclohexane-1-carboxylic acid
AcCl	acetyl chloride
AChE	acetylcholinesterase

AcOH	acetic acid
ADD-2004	2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic acid
ADHD	attention deficit hyperactivity disorder
ADHP	10-acetyl-3,7-dihydroxyphenoxazine
Aib	Aminoisobutyric acid
ALS	amyotrophic lateral sclerosis
Ar	aryl
ASA	amino salicylic acid
AT	ataxia-telangiectasia
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BPH	benign prostatic hyperplasia
br	broad
calcd.	calculated
COX	cyclooxygenase
Cpd	compound(s)
CTEIO	5-carboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl
CTMIO	5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl
CuAAC	copper catalysed azide alkyne cycloaddition
d	day or doublet
DABCO	1,4-diazabicyclo[2.2.2]octane
DCC	*N*,*N*'-dicyclohexyl carbodiimide
DCF	dichlorofluorescein
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DCM	dichloromethane
dd	doublet of doublet
DE	delocalization energy
dec.	decompose
DiE	dimerization energy
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMI	1,3-dimethyl-2-imidazolidinone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDC	*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EPR	electron paramagnetic resonance
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
FTIR	fourier transform infrared spectroscopy
GI	gastrointestinal
GO	graphene oxide
GSH	glutathione
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectroscopy
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
Lit.	literature
m	medium (FTIR) or multiplet (NMR)
*m*CPBA	*m*-chloroperoxybenzoic acid
MDMA	3,4-methylenedioxy-*N*-methylamphetamine
MeCN	acetonitrile
MeOH	methanol
min	minute(s)
MMT	[3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide]
Mp	melting point
MPH	methylphenedate
MPO	myeloperoxidase
MsCl	methanesulfonyl chloride
MTSL	methanethiosulfonate spin label
*n*BuLi	*N*-butyllithium
*n*Bulmi	*N*-butylimidazole
NMR	nuclear magnetic resonance
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
NSCLC	non-small cell lung cancer
O/N	overnight
OS	oxidative stress

PD	Parkinson's diseases
Pd/C	palladium on carbon
PG	prostaglandins
PGD_2	prostaglandin D_2
PGE_2	Prostaglandins E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
PH	pharmacophore hybridization
Ph	phenyl
PhD	doctor of philosophy
ppm	parts per million
QUT	Queensland University of Technology
Rh_2(S-DOSP)$_2$	etrakis[1-[[4-alkyl(C_{11}-C_{13})phenyl]sulfonyl]-(2*S*)-pyrrolidinecarboxylate] dirhodium(II)
ROESY	rotating-frame nuclear overhauser effect spectroscopy
ROS	reactive oxygen species
RT	room temperature
RTK	receptor tyrosine kinase
s	singlet or strong
SOD	superoxide dismutase
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl group
TBDMS-Cl	*tert*-butyldimethylsilyl chloride
TBTA	tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
*t*BuOH	*tert*-butanol
TEA	triethylamine
TEI	tetraethylisoindoline
TEIO	tetraethylisoindolin-2-yloxyl
TEMPO	2,2,6,6-tetramethylpiperidine-1-yloxyl
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yloxyl
THF	tetrahydrofuran
TLC	thin layer chromatography
TMI	1,1,3,3-tetramethylisoindoline
TMIO	1,1,3-tetramethylisoindolin-2-yloxyl
TMSCN	trimethylsilyl cyanide
TOAC	2,2,6,6-tetramethyl-*N*-oxyl-4-amino-4-carboxylic acid

TXA_2	thromboxane A
UC	ulcerative colitis
w	weak
wt./v	weight/volume

Statement of Original Authorship

The work contained in this thesis has not been previously submitted for an award at this or any other higher education institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where referenced or cited.

Signature: QUT Verified Signature

Date: July 2016

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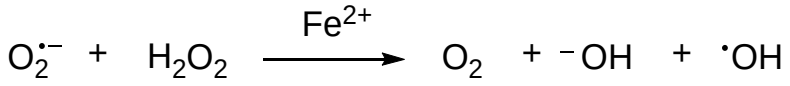
Chapter 1: Introduction

1.1 Free Radicals in Biology- Oxidative stress (OS)

Free radicals are essential components of chemical processes that take place in biological systems such as energetics, cellular metabolism and carcinogenesis.[1] Decades since it was first proposed, the Haber-Weiss reaction (**Scheme 1.1**) is thought to be an integral component of the mechanism underlying biological processes involved in both normal cellular function and disease related processes.[1-4]



Scheme 1.1. Harber-Weiss reaction.

Under normal physiological conditions, free radicals, mainly reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl radical), are associated with many cellular processes including cell-signalling pathways.[1,3,5,6] However, these processes require tight regulation of the production and bioactivity of reactive oxygen species (ROS) in the cell.[7]

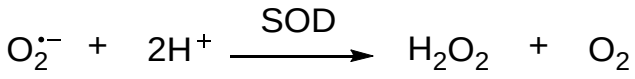
Abnormal levels of free radicals can cause significant damage to biomolecules.[4] Free radicals are involved in many chronic diseases and aging processes that affect the quality of life. Abnormal levels of free radicals result from exposure to environmental pollution, ultraviolet light and various illnesses.[1] The harmful state of abnormal ROS levels is known as *oxidative stress* (OS).[8] OS exerts its harmful effect in host cells by damaging cellular DNA, proteins, and lipids, thereby inhibiting their normal functions.[1] ROS are implicated in many pathological processes including cancer, schizophrenia, aging, Alzheimer's and Parkinson's diseases.[2,3,9]

For normal cell function, an equilibrium between ROS and the antioxidant defence system within cells is required. Thus, cells living under aerobic conditions are always faced with an oxygen dilemma - at physiological levels, they need oxygen to survive,

but toxic reactive oxygen species, which are generated from oxygen, can cause damage to the cells.

1.2 Endogenous Antioxidant Defence Systems

Under normal cellular redox equilibrium, cells are protected from ROS-induced damage by antioxidant defence mechanisms. Endogenous antioxidants either remove harmful oxidants (ROS) soon after they form, or repair damage caused by ROS.[10] They include components of membranes and DNA, enzymes and other radical scavenging molecules.[5] For instance, superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the decomposition of the superoxide radical into hydrogen peroxide (**Scheme 1.2**).[5,11] SOD-mimetic therapeutics show promising efficacy in disease states such as rheumatoid arthritis, reperfusion injury, inflammation and hyperoxia.[12]



Scheme 1.2. SOD catalyzed dismutation of the superoxide radical.

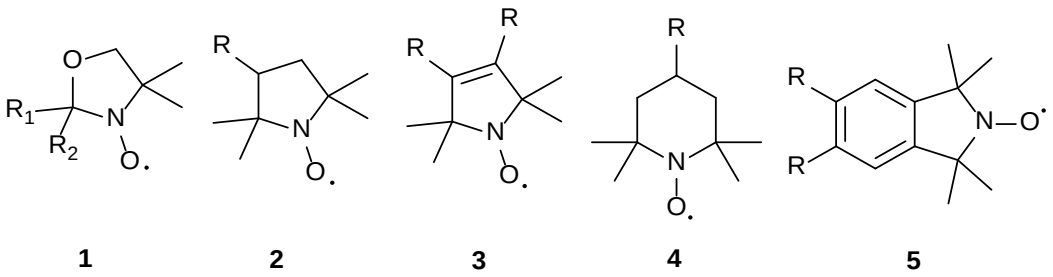
The “chain-breaking" antioxidants like vitamin E block free radical chain reaction processes that propagate the peroxidation cascade along a membrane.[7] Ascorbate (vitamin C) can reduce free radicals to form the water soluble dehydroascorbate. Glutathione (GSH), a versatile nucleophile and a reductant, can react with oxygen free radicals in various ways with transferases as a catalyst.[13-16]

To date, the search to develop potent antioxidant therapeutics is attracting more research interest because we have a better understanding of the mechanisms underlying free radical processes. Despite the progress, free radical induced tissue damage remains the primary mechanism that initiates and propagates many human pathophysiological conditions. One class of antioxidant compounds studied for their effect in both in biology and material science, is the stable nitroxide compounds.

1.3 Stable Nitroxide Radicals

Nitroxides are stable radical derivatives of nitrogen oxide that contain a disubstituted nitrogen atom linked to a single-valent oxygen atom.[17] The presence of the unpaired electron is the key paramagnetic property that allows the use of nitroxides as biophysical tools in electron paramagnetic (spin) resonance (EPR) spectroscopy.[9,18]

The most stable nitroxides occur in the form of sterically hindered cyclic compounds with oxazolidines, pyrrolidines, pyrrolines, piperidines and isoindolines among the most common ring classes of cyclic nitroxides (**Figure 1.1**). However, a significant number of acyclic forms also exist.[19]



1 **2** **3** **4** **5**

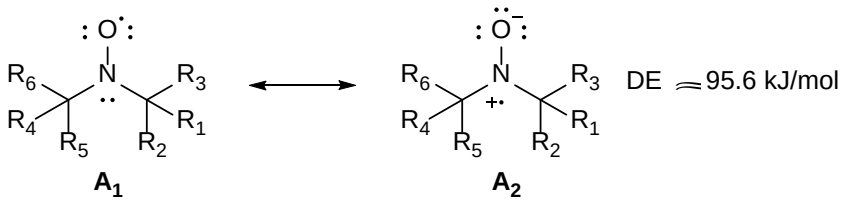
Figure 1.1. Common ring classes of stable nitroxide compounds: oxazolidine **1**, pyrrolidine **2**, pyrroline **3**, piperidine **4** and isoindoline **5**.

Despite the diverse nature of many of these structures, each stable nitroxide molecule has two common structural characteristics that influence its properties within organic synthesis, biomedicine and materials science: the effect of substituents in the immediate proximity of the nitroxide moiety and the resonance delocalization of the three-electron N-O bond.

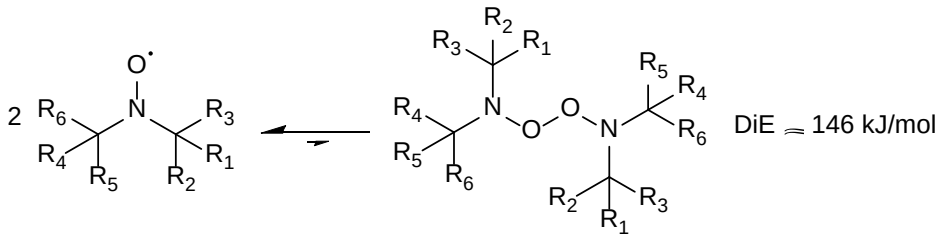
1.3.1 Stability of Nitroxide Radicals

Like most radical species, nitroxide free radicals have an open-shell electronic configuration with an odd number (single unpaired) of higher energy valence electrons. Though free radicals are generally highly reactive and short-lived, hindered nitroxide radicals are remarkably stable. This property has allowed organic chemists to synthesize, characterize and store them under normal laboratory conditions over long time without any extra precaution. Hence, a number of nitroxide

compounds are commercially available and have been used extensively as biophysical tools and in materials science.[18,20] Such exceptional stability is partly because of the strong delocalized three-electron bond within the radical centre, between the nitrogen and the oxygen atoms. In general, the spin density of the nitroxide moiety is slightly concentrated on the nitrogen atom.[21] The high delocalization energy within this bond increases the thermodynamic stability at the radical centre (**Scheme 1.3**).[22] This makes a pair of nitroxide radicals essentially unreactive toward one another. Thus, dimerization, a common radical-to-radical termination reaction, is highly unlikely to occur between nitroxide radicals (**Scheme 1.4**).[22,23]



Scheme 1.3. Resonance structures of the nitroxide moiety.



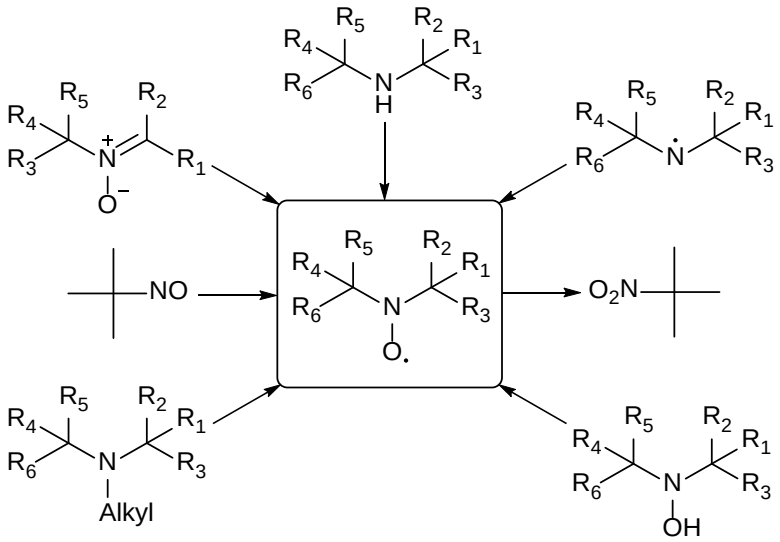
Scheme 1.4. Reversible dimerization of nitroxides.

Nitroxides are also stabilized significantly by steric hindrance and the polarity of substituents around the radical centre, particular at the α-carbon.[24-27] In general, saturating the α-carbon with bulky substituents confers kinetic stability at the radical centre. As a result, the prospect of many common radical mediated degradation pathways such as hydrogen abstraction is significantly diminished. As a practical example, tetraethyl substituted nitroxides are reduced more slowly by ascorbic acid than their tetramethyl counterparts.[28,29] Similar trends are observed in biological environments. Moreover, having an electron withdrawing group at the α-carbon increases the electron density of the oxygen atom on the resonance structure $\mathbf{A_2}$.[30]

This consequently increases the nucleophilicity of the oxygen atom, which in turn increases the ease of reduction of the nitroxide group.

1.3.2 General Synthetic Methodologies of Stable Nitroxides

Nitroxides are generated from a number of functional precursors (**Scheme 1.5**). However, stable nitroxides are prepared from the corresponding amines (secondary and tertiary) and hydroxylamines. These precursors undergo oxidation reactions in the presence of a number of oxidizing agents to form stable nitroxide radical-containing compounds.

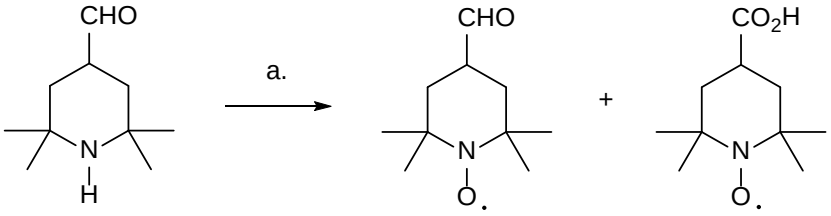


Scheme 1.5. Synthetic precursors for generating nitroxide radicals.

1.3.4.1. Oxidation of Secondary Amines

Oxidation of hindered secondary amines is the most common approach used to generate nitroxides. There are a variety of reagents currently employed to achieve this transformation. Amongst them, aqueous hydrogen peroxide is the main reagent used to oxidize secondary amines to nitroxides.[31] The reaction is performed in the presence of sodium tungstate and an inorganic weak base (e.g. sodium bicarbonate). There are also examples within the literature where sodium tungstate has been successfully replaced with phosphotungstic acid.[32,33] With either reagent, the reactions are usually carried out in water or aqueous methanol or acetonitrile at room temperature.

Despite being the most cost effective reagent of choice, hydrogen peroxide/sodium tungstate oxidation conditions require the use of excess reagents and longer reaction times. Such reaction conditions may affect the stability of other labile functional groups present. For instance, under such conditions, the oxidation of aldehyde **6** to the corresponding nitroxide **7** is accompanied by simultaneous oxidation of the aldehyde functional group to its acid derivative **8** (**Scheme 1.6**).[34] Limitations such as this can be overcome either by designing appropriate synthetic sequences for introducing the desired functionalities or by employing protecting group strategies.



6 **7** **8**

Scheme 1.6. Oxidation of amino formyl **6** to the corresponding nitroxide **7**. Reagents and conditions: a. H_2O_2, Na_2WO_4, $NaHCO_3$, MeOH (depending on the reaction time, ≥20% of compound **8** can be obtained).

Peracids, most commonly *m*-chloroperoxybenzoic (*m*CPBA) and sometimes *m*-nitroperoxybenzoic acid are the second most widely used reagents for oxidizing secondary amines in high yields.[35-37] Unlike the hydrogen peroxide-tungstate system, the peracid oxidation reactions are conducted in an organic solvent, mainly dichloromethane (DCM), with much shorter reaction times (30 mins to a few hours). The resulting *m*-chlorobenzoic acid by-product can be removed via extraction with base, or separated from the desired product by use of chromatographic techniques or trituration.

Generating nitroxides from secondary amines can also be achieved in high yields with at least two molar equivalent of dimethyldioxirane. This reagent was first investigated by Murray and co-workers when a variety of hindered secondary amines were each treated with 2 molar equivalents of dimethyldioxirane stock solution.[38] The authors proposed a reaction mechanism in which the amine is first oxidized to the hydroxylamine derivative by the one molar equivalent of the reagent, and the

second molar equivalent then easily oxidizes the resulting hydroxylamine to the desired nitroxide. A slight modification to the Murray procedure conducted by Brik involves an *in-situ* generation of dimethyldioxirane by reacting peroxomonosulfate with acetone under phase-transfer conditions in a biphasic media.[39] Both studies support the superiority of this reagent because of its versatility, shorter reaction time (30 minutes), convenience and high yields. With this method, the aldehyde nitroxide **7** in **Scheme 1.6** above was obtained directly from its amine precursor **6** in 95% yield.

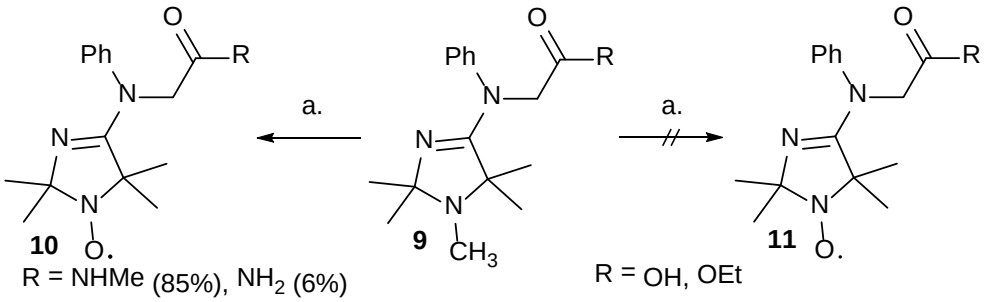
1.3.4.2. Oxidation of Tertiary Amines

Hindered tertiary amines are another type of functional group used as direct precursors for generating nitroxides. Although they are comparatively less common than their secondary amine counterparts, there are some examples in the literature where tertiary amines have been successfully oxidized to the corresponding nitroxides in moderate to high yields. The *N*-alkyl type tertiary amines are usually employed as protecting groups under incompatible reaction conditions with respect to either the nitroxide moiety, or other intermediate functionalities.

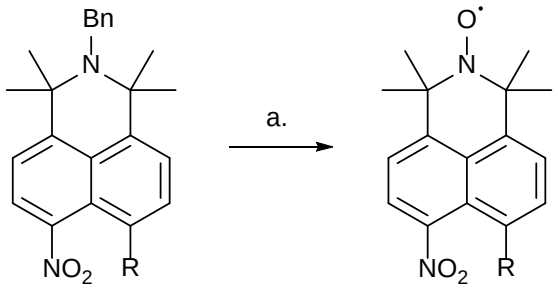
As with secondary amines, the H_2O_2/Na_2WO_4 oxidation system is used to convert some heterocyclic hindered tertiary amines into nitroxides.[40,41] One drawback to this method is the longer reaction times (days). There are a number of literature examples where this methodology has failed to yield the desired nitroxide product. For example, Grigor'ev *et al.* used this method to oxidize the amide version of *N*-methyl-3-imidazoline compound **9** to the nitroxides **10** but was unsuccessful with the carboxylic acid and ester derivatives **11** (**Scheme 1.7**).[40]

For the oxidation of *N*-benzyl type hindered tertiary amines to nitroxides, *m*CPBA is the reagent of choice. In **Scheme 1.8** below, the hindered *N*-benzyl azaphenalenes **12** were successfully oxidized to the corresponding nitroxides **13** with excess *m*CPBA.[42]

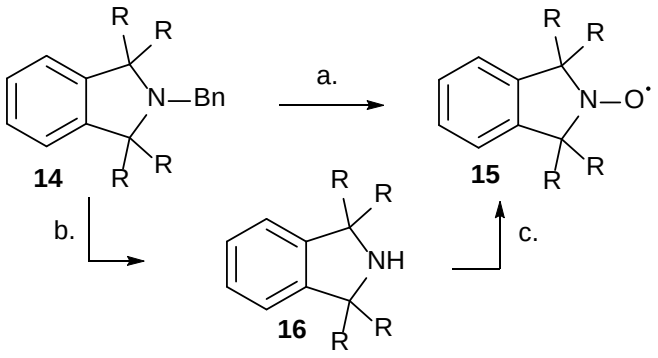
Even the more sterically hindered tetraphenylisoindoline compounds **14** were oxidized completely with *m*CPBA in high yield after six days (**Scheme 1.9.a.**).[43]



Scheme 1.7. Peroxy acid oxidation of *N*-methyl 3-imidazolines **9** to the corresponding nitroxides. Reagents and conditions: a. H_2O_2, Na_2WO_4, $NaHCO_3$.



Scheme 1.8. Peroxy acid oxidation of *N*-benzyl azaphenalenes to the corresponding nitroxides. Reagents and conditions: a. *m*CPBA, DCM, 1 d, R = H (90%), NO_2 (65%).

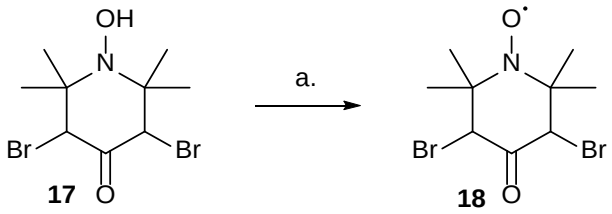


Scheme 1.9. Peroxy acid oxidation of *N*-benzylisoindolines to the corresponding nitroxides. Reagents and conditions: a. *m*CPBA, DCM, 1-6 d, R = Ph (71%), $(CH_2)_3Ph$ (54%); b. H_2, 10% Pd/C, R = Me (92%), Et (99%), *n*-Pr (83%), *n*-Bu (92%); c. *m*CPBA, DCM, 3 h, R = Me (91%), Et (90%), *n*-Pr (96%), *n*-Bu (99%).

Alternatively, the *N*-benzyl type tertiary amines **14** were reduced to the secondary amines **16** by hydrogenolysis. Then, the resulting secondary amines **16** were easily oxidized to the desired nitroxides **15** with the H_2O_2/Na_2WO_4 system or *m*CPBA (**Scheme 1.9.b.c.**).[44] This route gave higher yields over the two steps and the reaction times were reduced significantly.

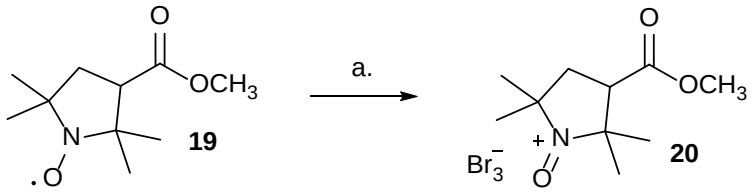
1.3.4.3. Oxidation of Hydroxylamines

Hindered hydroxylamines are the most easily oxidized precursors to nitroxides - so much so that it is highly unlikely that they are isolated in their pure form without their nitroxide derivatives as an impurity. Often, even brief exposure to oxygen in the atmosphere is enough to oxidize hydroxylamines to nitroxides.[45,46] There are some examples however, where the hydroxylamine derivatives are very stable and their oxidation requires more aggressive reaction conditions. For example, attempts to oxidize the dibromo hydroxylamine compound **17** with common oxidizing agents such as PbO_2, MnO_2, and H_2O_2/Na_2WO_4 proved unsuccessful. The oxidation was successful only when nitrous acid was employed (**Scheme 1.10**).[47]



Scheme 1.10. Nitrous acid oxidation of hydroxylamine **17** to the nitroxide **18**. Reagents and conditions: a. HNO_2.

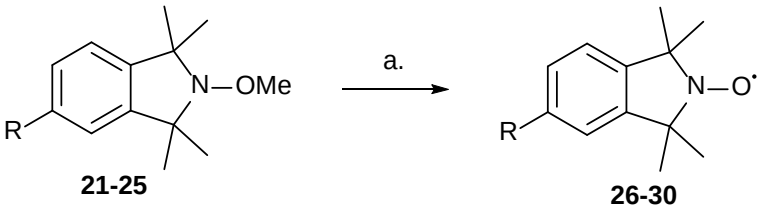
As a general rule, it becomes more difficult to oxidize hydroxylamine compounds when the number of electron withdrawing substituents around the radical centre increases. Some other metal salts have also been used as oxidizing agents for hydroxylamines.[48] Careful choice of oxidizing agent is required when oxidizing hydroxylamines to nitroxides. This is because certain oxidizing agents like Br_2 can further oxidize the desired nitroxide to the oxoammonium ion: with Br_2, the oxoammonium tribromide salt is formed (**Scheme 1.11**).[49,50]



Scheme 1.11. Bromine oxidation of nitroxide **19** to the oxoammonium salt **20**. Reagents and conditions: a. Br_2, -30 ^oC, quantitative.

1.3.4.4. Oxidation of Alkoxyamines

In controlled radical polymerization, homolytic cleavage of NO-C of alkoxyamines to generate nitroxides is common,[20,51,52] but synthetically, it is less common to synthesize nitroxides from alkoxyamines.[53] Recently, Bottle *et al.* examined the oxidative cleavage of a range of functionalized *N*-methoxy type alkoxyamines to generate the corresponding nitroxides.[54] The methoxyamine is a robust protecting group for the nitroxide moiety that can be used in synthesis and other comparative studies. Interestingly, they found that nitroxide compounds **26-30** could be generated quantitatively from the methoxyamine derivatives **21-25** with *m*CPBA as the oxidant under mild reaction conditions (**Scheme 1.12**).



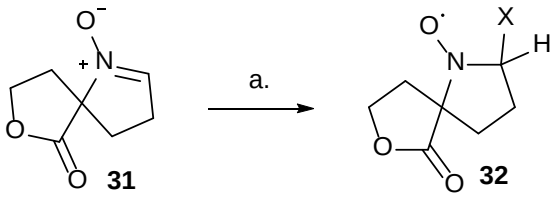
R = Me (**21, 26**), CHO (**22, 27**), Br (**23, 28**), NO_2 (**24, 29**), 9-Phenylanthracene (**25, 30**)

Scheme 1.12. *m*CPBA oxidation of *N*-methoxyamines to nitroxides. Reagents and conditions: a. *m*CPBA, DCM, 0 ^oC, 30 min -1 h; 70-90% yield.

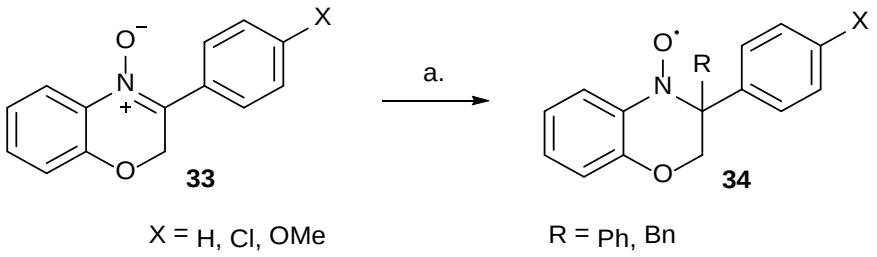
Complete conversion to the nitroxide was observed at room temperature within an hour. There are other examples in the literature where *m*CPBA was used to cleave the NO-C bond of a range of alkoxyamines during aldehyde synthesis. In these cases however, the nitroxides produced were unwanted by-products.[53,55-61]

1.3.4.5. Other Methods for Nitroxide Synthesis

Nitroxides are also generated from nitrones. Like nitroxides, nitrone compounds are regularly employed as spin traps for the identification of short-lived radicals including hydroxyl and carbon centred radicals.[62,63] In the process of capturing short-lived radicals, nitrones are converted to more persistent nitroxide radical species that are often analyzed by EPR spectroscopy. For instance, the spirolactonyl nitroxide spin adducts **32** were generated from the reaction of nitrone **31** with respective radicals (**Scheme 1.13**).[64] Also, when nitrones are alkylated with Grignard reagents, the resulting hydroxylamines can be oxidized in air, or in the presence of other oxidizing agents, to the equivalent nitroxides (**Scheme 1.14**).[65]



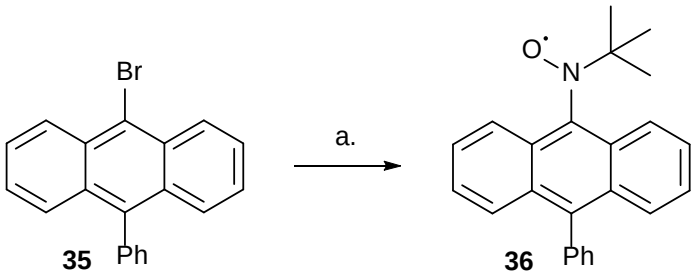
Scheme 1.13. Generation of nitroxides via radical spin trapping by nitrones. Reagents and conditions: phosphate buffer saline, H_2O_2, UV, and the respective radical sources $NaHCO_3$, Na_2SO_3, EtOH, $(t\text{BuO})_2$, KO_2 and DMSO.



Scheme 1.14. Generation of nitroxides via alkylation of nitrones with Grignard reagents. Reagents and conditions: RMgBr, THF, N_2, 1 h, then NH_4Cl, air, 46-82%.

Nitroxide compounds are also generated from nitroso compounds, mainly the tertiary aliphatic and aromatic nitroso compounds. As shown in **Scheme 1.15** below, when 9-bromo-10-phenylanthracene **35** is lithiated with *t*-butyllithium, the resulting anion was quenched with *t*-butylnitroso to give the hydroxylamine intermediate. The

hydroxylamine intermediate was then spontaneously oxidized in air to the corresponding nitroxide **36** in 52% yield.[66]

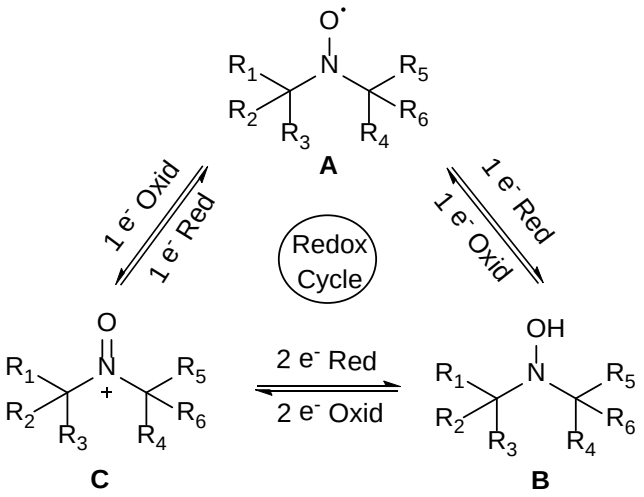


Scheme 1.15. Synthesis of nitroxides from tertiary nitroso compounds. Reagents and conditions: a. 1. *t*BuLi; 2. *t*BuNO; 3. Air, 52%.

1.3.3 Nitroxide Design and Applications

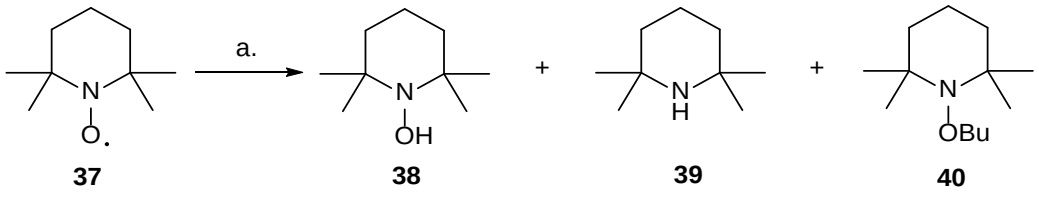
There are many literature reports that support the robust nature of the nitroxide moiety under diverse chemical reaction conditions. As the applications of nitroxides in biology and material science continue to expand, it is desirable to synthesize new nitroxide compounds that can be used to make functional materials. This section is aimed at highlighting synthetic transformations carried out within the past 10-15 years to design functional nitroxide compounds. The first consideration is that there are certain reaction conditions under which the nitroxide moiety can react. Other undesirable reaction conditions can produce side products, lower yields of the desired product, or stop product formation completely.

Reactions at the nitroxide radical centre, beside their ability to recombine with short-lived radical species, commonly involve a reversible redox triad between the nitroxyl radical **A**, its hydroxylamine **B** and oxoammonium ion **C** derivatives (**Scheme 1.16**).[67] In the presence of strong oxidizing agents, the nitroxide (**A**) undergoes a single-electron oxidation to its oxoammonium salt (**C**). The oxidation potential and stability of the resulting oxoammonium ion mainly depends on the nitroxide ring class and the surrounding substituents.



Scheme 1.16. Reversible redox cycle between nitroxide (**A**), hydroxylamine (**B**) and oxoammonium (**C**).

The nitroxide moiety is a weak oxidant in itself.[44] Hence, the nitroxide moiety is reduced readily to its hydroxylamine derivative by a variety of reducing agents including some metals, metal salts,[68] hydrazines,[69-71] ascorbic acid,[72-74] and hydrogen/catalyst. Because of the efficient reducing power and the strong nucleophilicity of hydrazines, it is not recommended, and is highly unlikely, to have a compound that contains both nitroxide and hydrazine functionalities. Most common metal hydrides have been employed successfully to reduce other functional groups in the presence of the nitroxide moiety.[75-77] This, however, depends on the nitroxide ring class. The reduction of a nitroxide to its hydroxylamine is reversible, oxidation of the hydroxylamine back to the nitroxide is often achieved by exposing it to air. Organometallic reagents can also reduce nitroxides. The products isolated when the piperidine nitroxide **37** (TEMPO) was treated with *n*-butyllithium included the hydroxylamine **38**, amine **39** and alkoxyamine **40** derivatives (**Scheme 1.17**).[78,79]



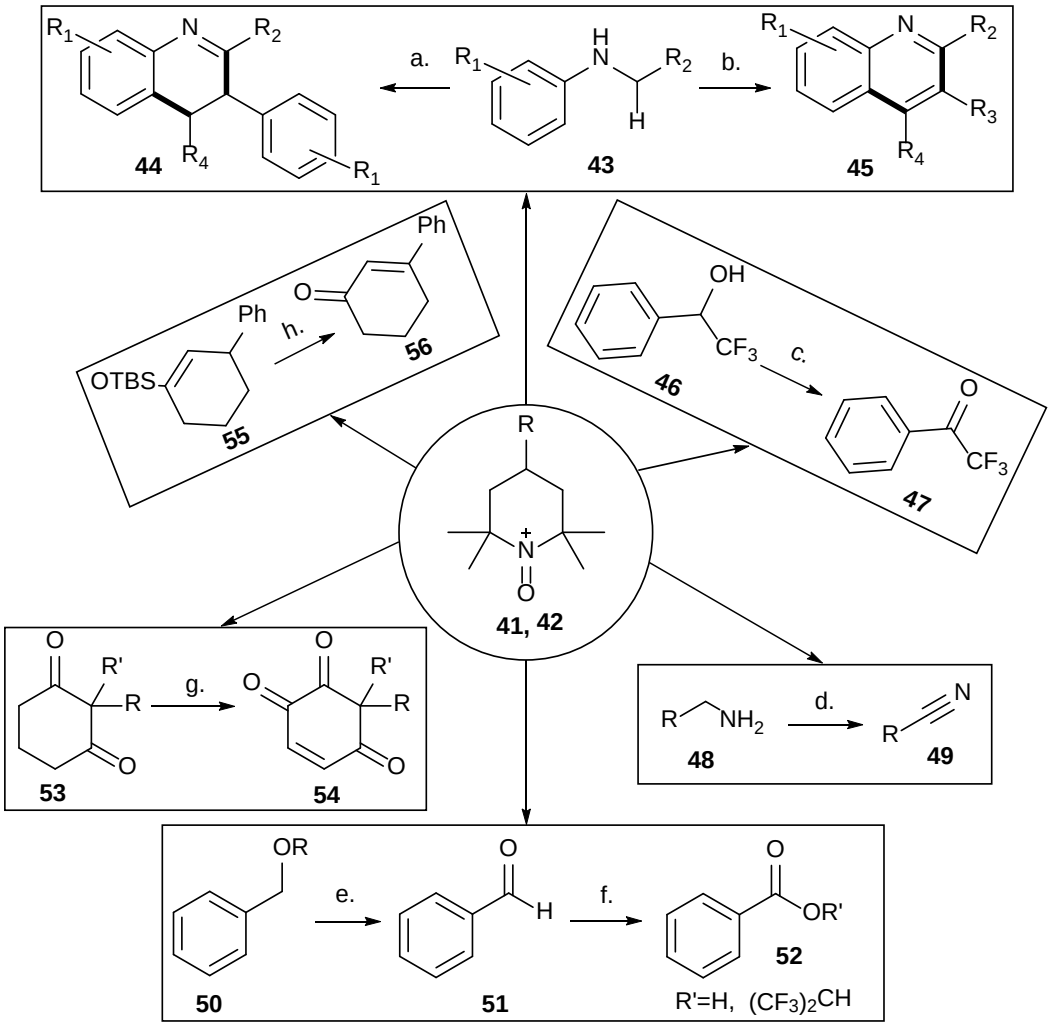
Scheme 1.17. Lithiation of nitroxides. Reagents and conditions: a. *n*BuLi.

Because of the weak basic character of the nitroxyl group (pK_{H+} = -5.8),[80] nitroxides disproportionate in strong acid media to form the oxoammonium ion and the protonated hydroxylamine.[25,81-83] The reversibility of the acid catalyzed disproportionation process depends on the pH and the excess acidity function. In concentrated strong acid media, the equilibrium is completely shifted toward the oxoammonium salt and the protonated hydroxylamine, but the nitroxide is usually regenerated if such concentrated acid media is diluted, neutralized or basified.[25]

Most oxoammonium ions are unstable and highly reactive towards other functional groups.[84,85] The oxoammonium ion is a strong oxidizing agent. It is used in catalytic or stoichiometric amounts to oxidize a number of substrates under acidic, neutral or basic conditions.

In **Scheme 1.18** below, the oxoammonium salts of TEMPO and 4-acetylamino-TEMPO derivatives (compounds **41** and **42**) oxidize alcohols to aldehydes,[86] ketones,[87-89] esters,[90] or carboxylic acids;[86] aldehydes to esters[91] or carboxylic acids;[86] diketones to triketones;[92] primary amines to nitriles;[93] *N*-alkylanilines to dihydroquinazolines and quinolones;[94,95] and perform oxidative cleavage of silyl enol ethers[89] and benzylic related ethers.[96]

Evidently, being aware of the reaction conditions under which the nitroxide moiety can react and its functional group tolerance characteristic could be helpful in the design and synthesis of functional nitroxide compounds.



Scheme 1.18. Synthetic applications of oxoammonium salts. Reagents and conditions: a. $Fe(OTf)_2$, DCM, 60 ^oC, 24 h, 15 examples, 30-70%; b. $FeCl_3$, appropriate styrene, DCM, 60 ^oC, 24 h, 15 examples, 30-86%; c. 2,6-lutidine, DCM, RT, 20 examples, 49-99%; d. Pyridine, DCM, RT, 15 examples, 75-95%; e/g. MeCN, 10 examples each, 36-93%; f. $(CF_3)_2CHOH$, pyridine, DCM, RT, 26 examples, 68-96%; h. 2-azaadamantane-*N*-oxyl oxoammonium ion, MeCN, -78 ^oC, 1 h, 16 examples, 6-88%; (R = H (**41**), NHAc (**42**)).

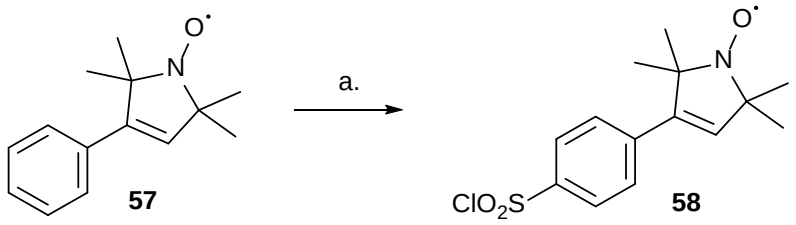
1.3.4 Functional Nitroxides

When functionalizing a molecule containing the nitroxide moiety, the process is not restricted to reactions conducted only in the presence of the radical species. In many instances, functional groups are introduced in the molecular framework of nitroxide precursors like amines or hydroxylamines. In other instances convenient protecting

groups, such as the acetyl-protecting group, can be used to mask the radical centre if necessary.

Aromatic substitution reactions are synthetic transformations commonly used in organic chemistry. Hence functionalizing nitroxide compounds with a range of aromatic substitution reaction conditions is essential.

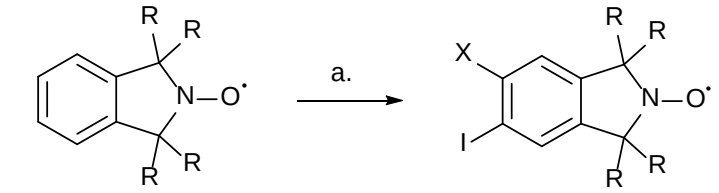
In the synthesis of the terminal acetylenic sulfone linked to a pyrroline nitroxide, the intermediate nitroxide sulfonyl chloride **58** (**Scheme 1.19**) was obtained after electrophilic aromatic sulfonation of **57** with chlorosulfonic acid at 0 ^{o}C.[97] Because of the strong acid content of the reaction media, PbO_2 was employed to oxidize any pre-formed hydroxylamine (by acid disproportionation of nitroxide) back to the target nitroxide.



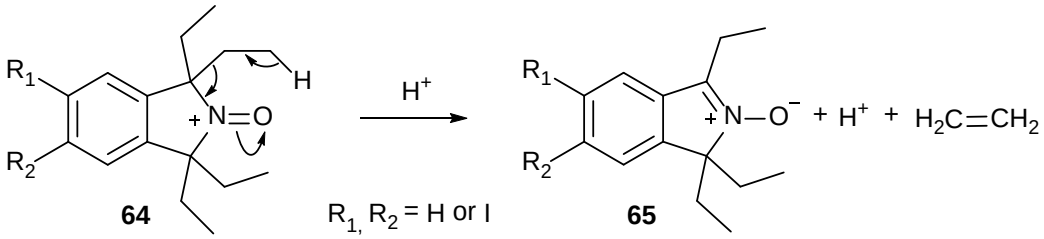
Scheme 1.19. Electrophilic aromatic sulfonation of nitroxide **57**. Reagents and conditions: a. HSO_3Cl, 0 ^{o}C to RT, 2 h, then PbO_2, O_2, 10 min, 73% yield.

The isoindoline class of nitroxides are readily functionalized on the aromatic ring, mainly at the 5- and 6-positions. Like most aromatic compounds, introducing a halide group on the aromatic ring can increase the synthetic versatility of the substrate. Whereas direct ring bromination (with both single or double substitution available at the 5 and 6 positions) has been shown in a number of examples in the literature,[98] it is only recently that the direct iodination of isoindoline nitroxides was established. Di-iodination of both tetramethyl and tetraethyl substituted isoindoline were successfully achieved with periodic acid and potassium iodide as a source of a strongly electrophilic iodonium ion, in concentrated sulfuric acid (**Scheme 1.20**).[99] For tetraethyl isoindoline nitroxide, the authors reported the isolation of 13% of iodinated nitrones **65** (**Scheme 1.21**). The nitrones **65** formation was attributed to the decomposition (acid-catalyzed disproportionation) of the preformed oxoammonium

ions **64** under such strong acidic reaction conditions. Under the same reaction conditions, selective single iodidations were accompanied by the formation of the small amount of the di-iodo counterparts. The products were separated by flash column chromatography. These reaction conditions were also used on the secondary amine precursors successfully.



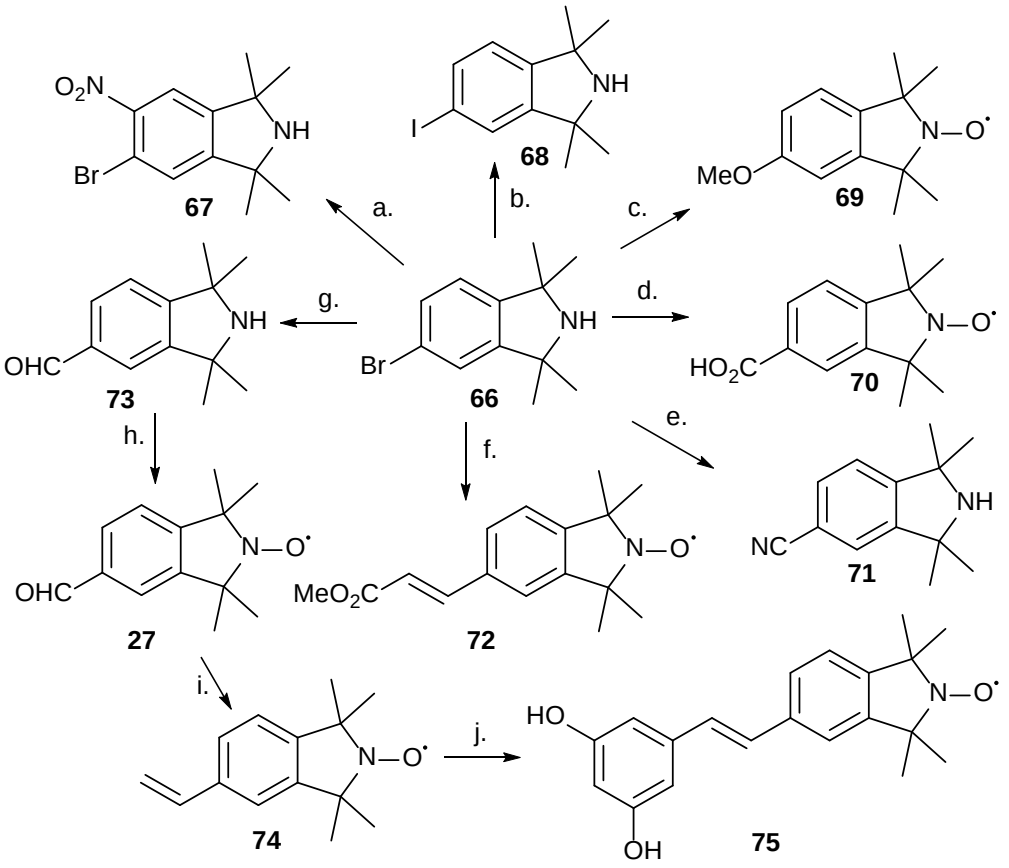
Scheme 1.20. Direct iodination of isoindoline nitroxides. Reagents and conditions: a. H_5IO_6, KI, conc. H_2SO_4, 0 °C to RT. 3 h, **61** (48%); **62** (82%); **63** (37%).



Scheme 1.21. Acid-catalyzed decomposition of tetraethylisoindoline oxoammonium salts to nitrones.

The 5-bromoisoindoline compound **66** is a versatile nitroxide precursor used in a variety of synthetic transformations. **Scheme 1.22** displays some useful synthetic transformations carried out with compound **66**. The reactions include formylation,[100] cyanation,[101] iodination,[102] nitration,[103] carboxylation,[100] copper-catalyzed nucleophilic methoxylation,[42] palladium-catalyzed and Heck coupling reactions.[104] The intermediate dianion formed when compound **66** is treated with two molar equivalents of *n*-butyllithium can be quenched with iodine, dry CO_2, or DMF to give the iodo **68**, carboxylic acid **70**, or aldehyde **73** derivatives respectively. In the synthesis of potential antioxidant isoindoline nitroxide-resveratrol analogue **75**, the aldehyde nitroxide **27** was subjected to a Wittig reaction with

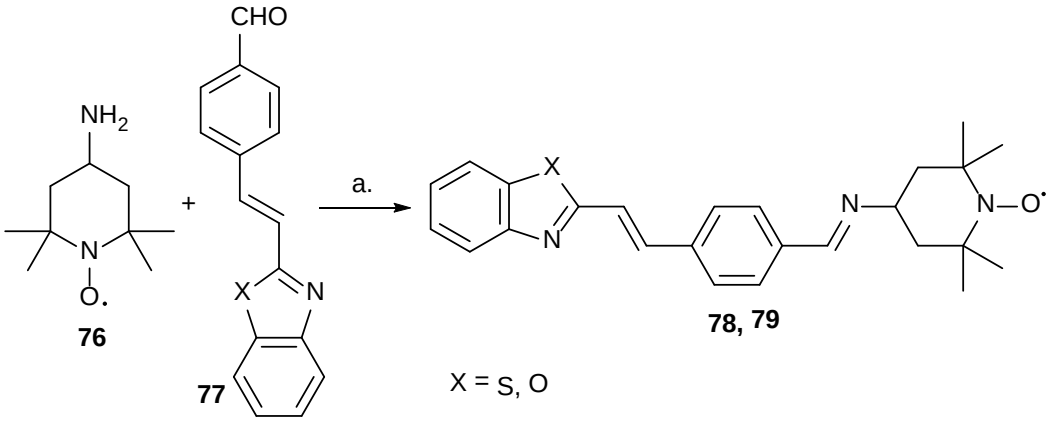
triphenylmethylphosphonium iodide in the presence of K_2CO_3, KOH and 18-crown-6 in refluxing dioxane to give the styrene nitroxide derivative **74**.[105]



Scheme 1.22. Synthetic utility of bromoamine precursor **66**. Reagents and conditions: a. HNO_3, H_2SO_4, 0 °C to RT, 3 h, 56%; b. *n*BuLi, THF, -78 °C, then I_2, 70%; c. *m*CPBA, DCM, 3 h, RT, then NaOMe, CuI, EtOAc, MeOH, reflux, 16 h, 88%; d. *n*BuLi, THF, -78 °C, then CO_2, then H_2O_2, $Na_2WO_4.7H_2O$, $NaHCO_3$, 62%; e. CuI, *n*Bulmi, $K_4[Fe(CN)_6]$, *o*-xylene, 180 °C, 4 d, 78%; f. K_2CO_3, methyl acrylate, $Pd(OAc)_2$, PPh_3, DMF, 120 °C, 72 h, 62%; g. *n*BuLi, THF, -78 °C, then DMF, 100%; h. *m*CPBA, DCM, 72%; i. PCH_3Ph_3I, K_2CO_3, KOH, 18-crown-6, dioxane, reflux, 48 h, 62%; j. KOAc, 5-iodo-1,3-phenylene diacetate, $Pd(OAc)_2$, Bu_4NBr, DMF, 80 °C, 10 h, then NaOMe (0.1 equiv), MeOH, 1 h, RT, 78%.

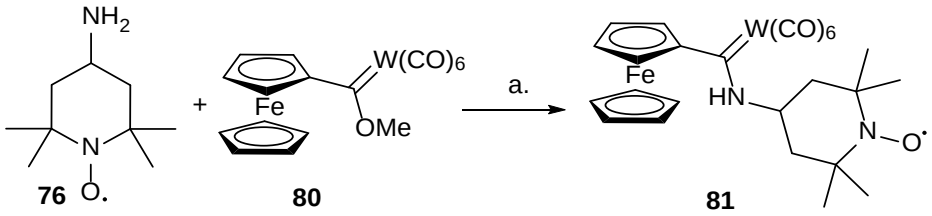
Piperidine type nitroxides are commonly functionalized at the 4-position. Two stable Schiff base piperidine nitroxide derivatives of benzothiazole and benzoxazole-based dyes (**78** and **79**) were synthesized in high yields when 4-amino-TEMPO **76** was

reacted in an alcohol solution with compound **77** in the presence of a catalytic amount of acetic acid (**Scheme 1.23**).[106]



Scheme 1.23. Synthesis of Schiff base piperidine nitroxide derivatives. Reagents and conditions: a. AcOH, EtOAc, reflux, 2 h; **78** (X = S, 83%) and **79** (X = O, 81%).

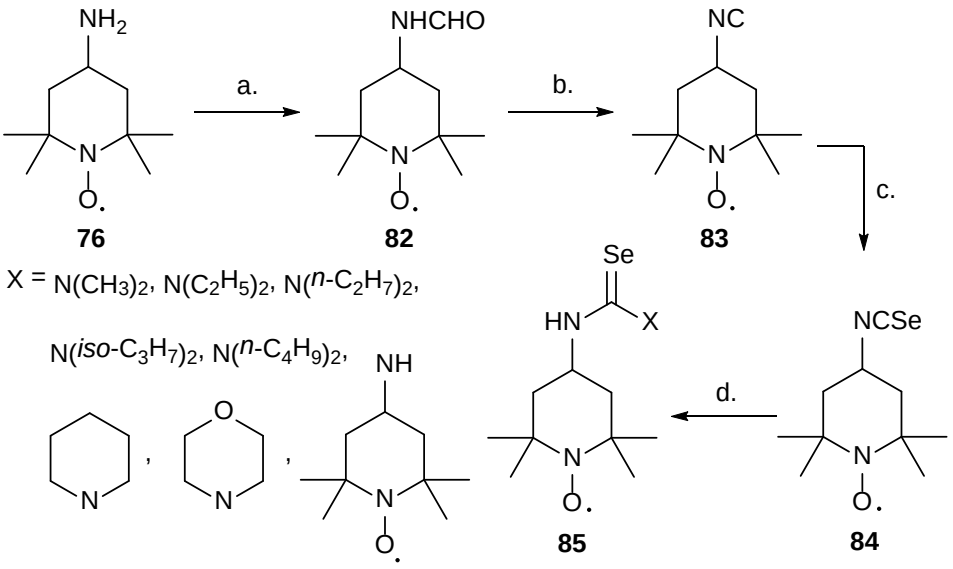
Nitroxides have also been incorporated into Fischer-type carbene complexes to give novel organometallic nitroxide spin systems (**Scheme 1.24**).[107] Aminolysis of ferrocenyl methoxy carbene **80** with excess 4-amino-TEMPO **76** afforded the TEMPO-linked ferrocenyl methoxy carbene complex **81**. Complex **81** is quite stable and contains two redox systems, namely the nitroxide and ferrocenyl groups. Thus, complex **81** could be further explored as potential antioxidant therapeutics or for use in charge-storage devices.



Scheme 1.24. Synthesis of 4-amino-TEMPO-linked ferrocenyl methoxy carbene complex **81**. Reagents and conditions: a. THF, RT, 2 d, 58%.

Organoselenium compounds are useful building blocks for functional materials and as potential pharmaceutical agents. In the synthesis of some functionalized selenium-nitroxide compounds, 4-amino-TEMPO **76** was successfully transformed to a range of selenoureas and selenocarbamates (**Scheme 1.25**).[108] The transformation involved

the reaction of **76** with excess ethyl formate to give the 4-aminoformyl derivative **82**. Then, compound **82** was dehydrated with gaseous phosgene to give the 4-isocyano derivative **83**. Selenation of **83** with metallic selenium gave the 4-isoselenocyanato **84**, which was subsequently reacted with a series of amines and alcohols to give selenoureas and selenocarbamates **85**.

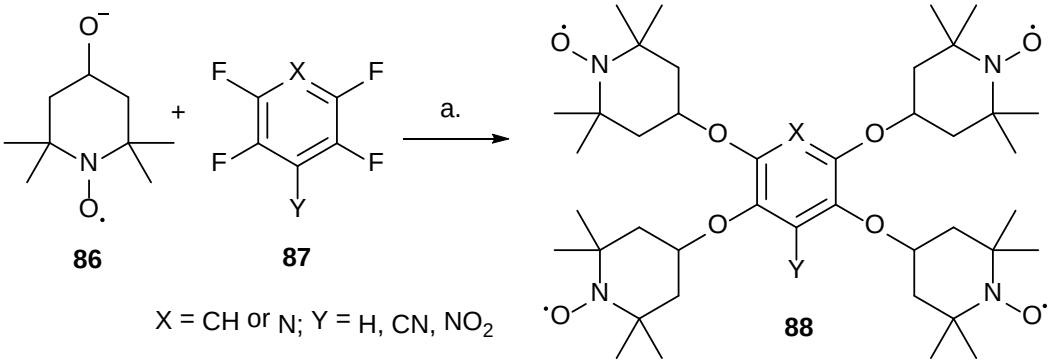


Scheme 1.25. Synthesis of organoselenium nitroxides. Reagents and conditions; a. HCO_2Et, reflux, 5 h, (96%); b. $COCl_2$ or $ClCOOCCl_3$, TEA, DCM, 0 ^oC, (78%); c. Se, $CHCl_3$, 61 ^oC 70 h, 38%; d. HX/NaX, benzene, 19-87%.

Recently, Turro and co-workers reported a synthetic procedure for making multi-spin systems via nucleophilic aromatic substitution of fluorides with the alkoxide anion of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yloxyl (TEMPOL) **86** (**Scheme 1.26**).[109] With a range of multi-fluorinated aromatic cores **87**, the authors selectively introduced mono, di, tri and tetranitroxides **88** under appropriate reaction conditions. In contrast to mononitroxide compounds, multi-spin nitroxide compounds like **88** have good electron-electron spin-exchange between individual nitroxide moieties. This enables their use as efficient paramagnetic relaxation agents and electron spin agents for dynamic nuclear polarization experiments.

Copper-catalyzed azide alkyne cycloaddition (CuAAC) reactions are another useful synthetic approach currently employed to produce functionalized nitroxides. **Figure 1.2** shows a few stable azide and acetylene nitroxides synthesized and successfully

used in CuAAC click reactions for the synthesis of a range of triazole-functionalized nitroxide compounds.[97,110-114]



Scheme 1.26. Synthesis of tetranitroxides **88** via nucleophilic aromatic substitution of fluorides. Reagents and conditions; a. DMI, $NaNH_2$, 0 ^oC-RT-80 ^oC, 37-81%.

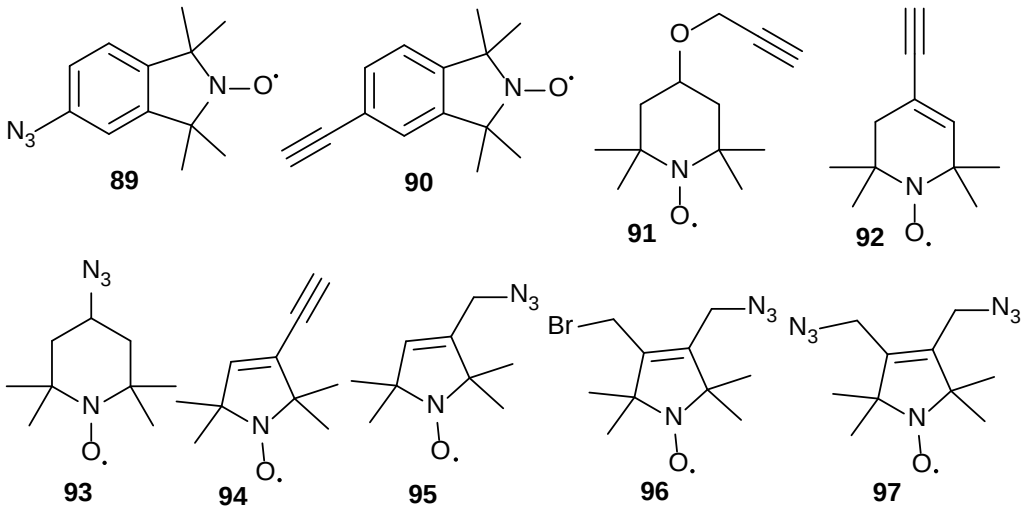
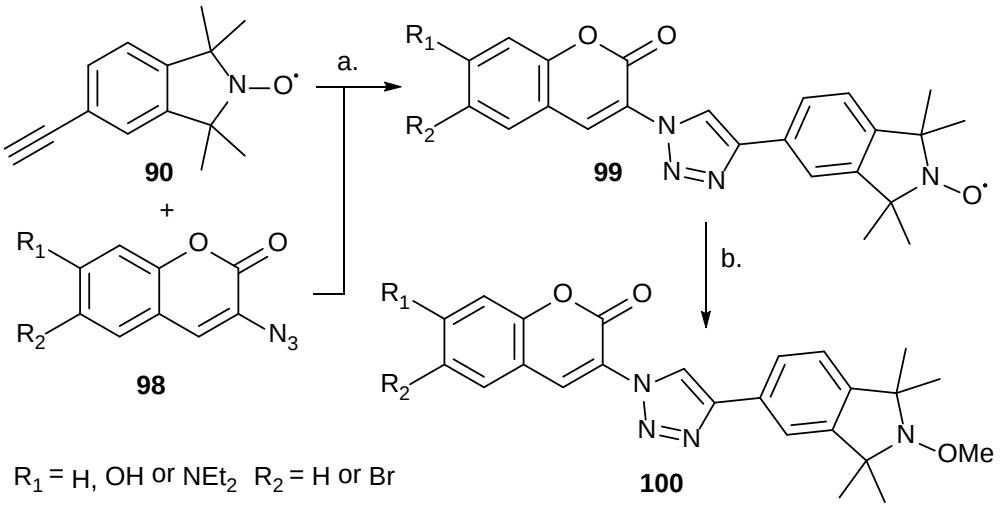


Figure 1.2. Common azide and acetylene nitroxides.

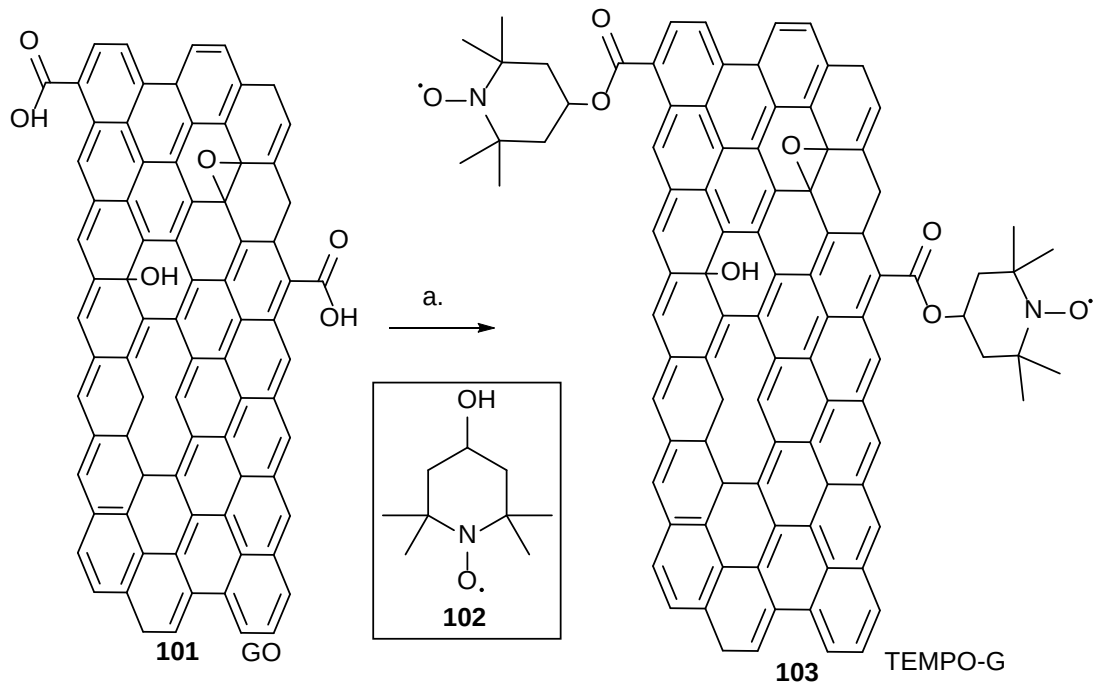
Compound **99**, a triazole coumarin-based profluorescent nitroxide which significantly suppressed fluorescence, was synthesized via a copper-catalyzed azide alkyne 1,3-dipolar cycloaddition reaction of 3-azido-7-diethylamine coumarin **98** and alkyne TMIO (tetramethylisoindolin-2-yloxyl) **90** (**Scheme 1.27**).[111] The fluorescence of the coumarin fluorophore was restored when **99** was converted to its alkoxyamine derivative **100**.



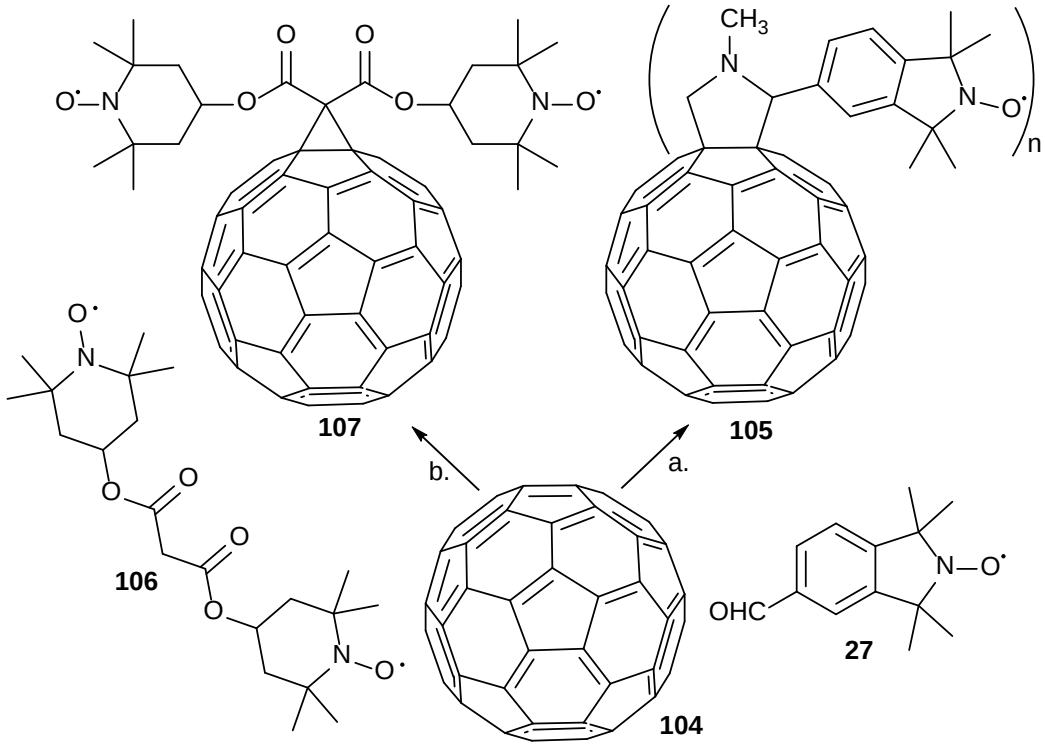
Scheme 1.27. Copper-catalyzed azide alkyne cycloaddition of nitroxides. Reagents and conditions: a. $CuSO_4$, NaAsc, $EtOH/H_2O$, 16 h, RT, 60-90%; b. H_2O_2, $FeSO_4.7H_2O$, DMSO, 30 min, 78-90%.

A number of examples of nitroxides grafted to carbon-based surfaces to give carbon-polymer composite materials have been reported. In an attempt to design higher capacity next generation lithium ion batteries, graphene **101** (graphene oxide, GO) was grafted with TEMPOL **102** via the conventional esterification of its carboxylic acid groups (**Scheme 1.28**).[115] The resulting hybrid nitroxide-graphene anode conjugate material **103** possesses superior lithium storage capacity because it has a dual redox system that comprises of graphene and the reversible redox cycling nitroxide.

Yan and co-workers also grafted isoindoline nitroxide to fullerene (C_{60} and C_{70}) cages.[116] In **Scheme 1.29** below, the nitroxide-linked nanoparticle **105** (TMIO-C_{60}) was prepared by refluxing fullerene **104** and formyl TMIO **27** in the presence of sarcosine. Along the same line, Bingel–Hirsch reaction of fullerene **104** and the malonate ester nitroxide **106** afforded the nitroxide methanofullerene derivative **107**.[117]

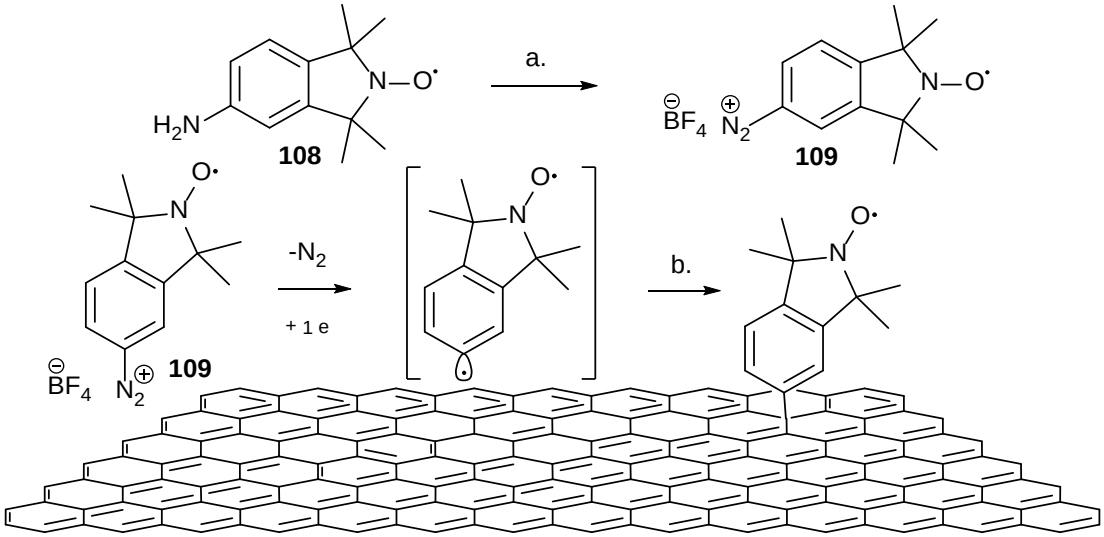


Scheme 1.28. Grafting of nitroxides to graphene surfaces. Reagents and conditions: a. $SOCl_2$, **102**, DMF, 70 °C, 24 h; then TEA, 100 °C, 60 h, $NaHCO_3$.



Scheme 1.29. Grafting of nitroxides to fullerene cages. Reagents and conditions: a. Sarcosine, **27**, toluene, reflux, 20 h; b. CBr_4, 94, DBU, toluene, 25 °C.

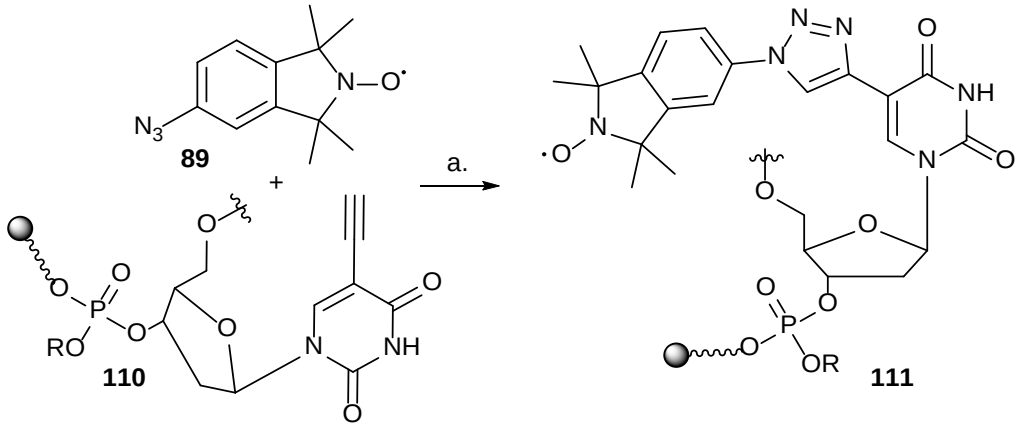
Blinco *et al.* synthesized the novel diazonium-based isoindoline nitroxide **109** from its 5-amino-TMIO precursor **108** (**Scheme 1.30**) and grafted it to a number of different carbon allotropes (single and multi-walled carbon nanotubes, graphene, glassy carbon and carbon fibre).[118] This was achieved by either an electrochemical reduction of the diazonium or, for graphene and carbon nanotubes, spontaneous reaction.



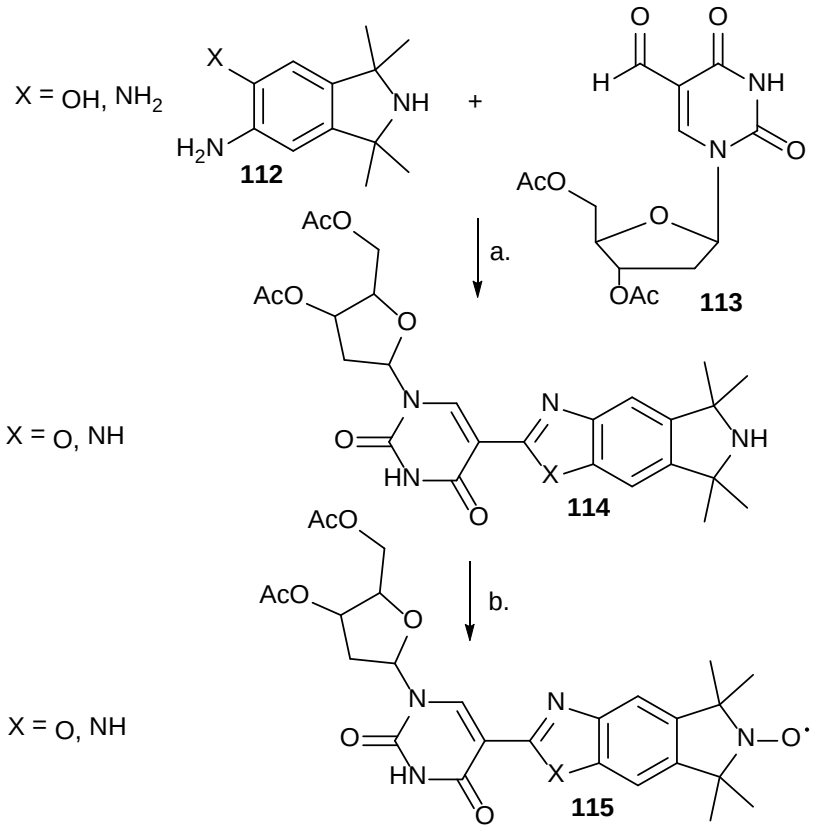
Scheme 1.30. Grafting of diazonium-based nitroxide to carbon allotropes. Reagents and conditions: a. $NOBF_4$, MeCN; b. grafting of diazonium **109**.

Incorporating nitroxides into complex biomolecules is an effective technique commonly used to obtain useful structural information. Recently, isoindoline based nitroxides were synthesized and incorporated into duplex DNA as spin labels. In one instance, CuAAC of an azide functionalized isoindoline nitroxide **89** with 5-ethynyl-2′-deoxyuridine oligomers **110** afforded the triazole-linked spin labelled oligonucleotide derivatives **111** (**Scheme 1.31**).[113]

Along the same line, novel isoindoline nitroxide spin-labelled benzimidazole and benzoxazole nucleosides **115** were successfully synthesized when 3',5'-di-*o*-acetyl-5-formyl-2'-deoxyuridine **113** was reacted with isoindolines **112** in the presence of potassium ferricyanide, followed by oxidation of the resulting amine intermediate **114** (**Scheme 1.32**).[119] These nitroxide-based nucleosides **115** were incorporated into DNA oligonucleotides and they displayed limited mobility in the duplex DNA.



Scheme 1.31. Synthesis of triazole-linked nitroxide-oligonucleotide spin label **111** Reagents and conditions: a. TBTA, CuBr, *t*BuOH/DMSO; NH_4OH, 55 ^oC, 100%.



Scheme 1.32. Synthesis of triazole-linked nitroxide-benzimidazole and benzoxazole-based spin label nucleosides. Reagents and conditions: a. $K_3Fe(CN)_6$, MeOH, 40% (X = O); Iodobenzene diacetate, 47% (X = NH); b. *m*CPBA, 50-56%.

In general, the structure and paramagnetic properties of nitroxide containing compounds accounts for their wide use in biology and materials science. With better

understanding of the chemistry of stable nitroxides, a variety of synthetic transformations have been used to design novel functional nitroxide compounds. Recently, the therapeutic application of stable nitroxide compounds as potent antioxidants has gained significant research interest and is quite promising.

1.3.5 Antioxidant Nitroxides

Although stable nitroxide compounds have been used for many years as biophysical tools, it is only within the past two decades that their therapeutic applications have been explored.[6,7,13,19,112,120-130] Stable nitroxides have antioxidant actions and thus protect biological tissues against oxidative stress.[131] The antioxidant action of nitroxides is mainly attributed to the redox cycle (**Scheme 1.16**) that involves the nitroxide and its oxoammonium ion and hydroxylamine derivatives. In cellular environments, the equilibrium of this redox triad depends mainly on the redox-status of the surrounding cellular components.

As stable free radicals, nitroxides directly scavenge ROS type radicals that cause damage to biomolecules.[132,133] pH-responsive TEMPAMINE-linked nanoparticles **116** scavenge ROS and offer renal protection in ischemia-reperfusion acute kidney injury model in mice (**Figure 1.3**).[132]

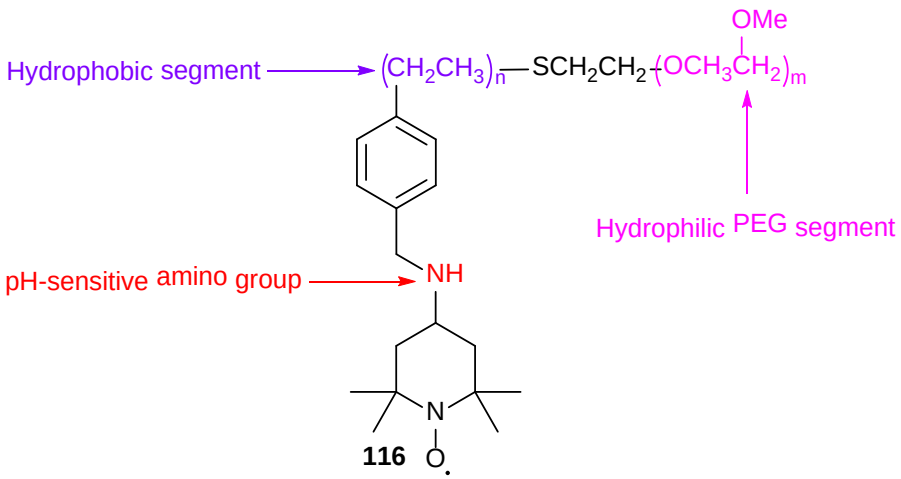
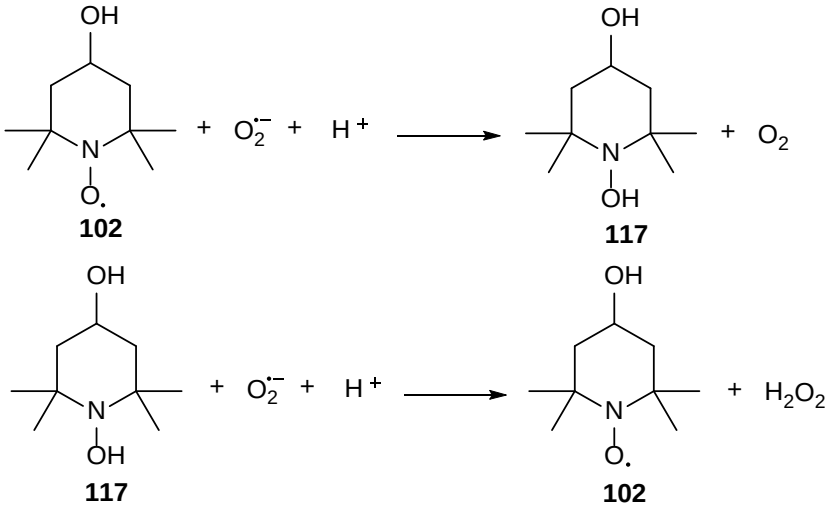


Figure 1.3. ROS scavenging pH-responsive TEMPAMINE-linked nanoparticles **116**.

Under aerobic conditions, the hypoxanthine/xanthine oxidase system can generate high levels of superoxide radicals and H_2O_2 which can induce damage to a cell.[134]

When Chinese hamster V79 lung fibroblasts were exposed to hypoxanthine/xanthine oxidase, a dose-dependent decrease in survival of V79 cells was observed.[135] However, in the presence of several nitroxide compounds, the V79 cells survived the cytotoxicity induced by the hypoxanthine/xanthine oxidase-derived oxidative stress. In comparison to endogeneous antioxidants such as SOD, the V79 cell protection effect of TEMPOL **102** was superior.

Nitroxides also possess a metal-free SOD-mimetic action; i.e. they catalyze the dismutation of superoxide radicals.[16,136,137] In *in vitro* and *in vivo* studies, TEMPOL **102** appeared to protect mammalian cells against oxidative damage, both by an SOD-mimetic action.[138,139] In **Scheme 1.33** below, the nitroxide TEMPOL **102** and its hydroxylamine derivative TEMPOL-H **117** can both offer protection against cytotoxic oxidants via redox processes in which the nitroxide can be regenerated.



Scheme 1.33. SOD mimetic-like action of TEMPOL **102**.

As stable free radicals, nitroxides also exert their antioxidant action by inhibiting lipid peroxidation process. Lipid peroxidation is a common free radical related process by which damage to tissues occurs.[123,125,129] Lipid peroxidation modifies lipid-containing biological macromolecules such as proteins and membrane lipid bilayers to produce toxic pro-oxidants.

In addition to inducing chemical change in lipid membranes and proteins that compromises their biophysical properties, free radicals and ROS can damage

sensitive neurones. Although the mechanism underlying most neurodegenerative diseases is not fully understood, clearly oxidative stress is involved in the pathway through which neuronal damage occur.[140-143] Nitroxides have also been reported to offer neuroprotection to neuronal cells that are under oxidative stress associated with neurodegenerative diseases.[144,145] In *in vitro* Parkinson's disease studies, nitroxides were reported to protect dopaminergic neurons from 6-hydroxydopamine-induced apoptosis.[146]

Stable nitroxide compounds have also been reported to possess anti-cancer activities. Such promising redox-chemotherapeutic effects have been associated with the ability of the nitroxide radical to disrupt some tumor related redox chemistry.[122,147-149] Ataxia-telangiectasia (AT) is a genetic disorder that is commonly associated with cancer predisposition, immunodeficiency, growth retardation, infertility, neurological degeneration and sensitivity to ionizing radiation. When the tumor prone AT-mutated (*Atm*)-deficient mice were fed high dose of nitroxides, Wynshaw-Boris and co-workers observed that the nitroxides significantly increased the lifespan of the mice by delaying the start of thymic lymphomas.[149] The *Atm*-deficient mice are a murine model for the human disease AT. Such anti-cancer activity of nitroxides was attributed to the antioxidant action in reducing oxidative stress.

Recently, some stable nitroxide compounds were shown to inhibit the action of the myeloperoxidase (MPO)-mediated hypochlorous acid production system.[130] The MPO system is implicated in a number of inflammatory diseases. The inhibitory action of nitroxides involves a competing one-electron oxidation of nitroxides by the so-called compound **I** state of MPO which subsequently prevents the oxidation of chloride, and the MPO reduction by superoxides. As the number of disease states increases where nitroxides display therapeutic value, there is a need for the design and synthesis of novel antioxidant nitroxides that are based on specific biological targets.

Stable antioxidant nitroxides have also been reported to possess promising radioprotective, antihypertensive, anti-aging, anti-inflammatory, and weight control activity.[122,150] Such widespread applications of stable nitroxides make the effort to design and synthesize novel antioxidant nitroxides a key area of current research

interest. Thus, a rational drug design and development strategy could be used as an attractive approach for the development of novel nitroxide-based antioxidant therapeutics.

1.4 Modern Drug Discovery

At a cellular level, the human body continuously undergoes countless complex chemical reactions. These chemical reactions regulate our growth, maintenance and daily activities but they are also involved with the changes in cellular functions associated with disease conditions. For instance, chemical imbalance can trigger a cascade of undesired events that disturb the normal function of a specific protein which could lead to a disease state. Drugs administered to manage disease conditions often work by interacting with endogenous target molecules to either restore a balanced physiological state or reverse the disease state. The process and effort to develop drugs on as rational a basis as possible, that minimizes trial-and-error factors, is known as drug design.[151]

Advances in modern drug discovery are a product of collective research effort from government, academia, and pharmaceutical industries. The modern drug discovery process is an interdisciplinary endeavour that involves clinicians, pharmacologists, biochemists and medicinal chemists.[152,153] Modern techniques such as high-throughput screening methods, combinatorial chemistry, rapid DNA sequencing and new computing methods are used to identify promising “lead” compounds. This has led to the development of a number of successful drug candidates in modern medicine.[154] However, the modern drug discovery and development process remains challenging. The process of discovering and developing a drug and releasing it to the market takes approximately 15 years and costs up to $2 billion.[152,155-157] The success rate of this process is low with only one in every 5,000-10,000 developed drug candidates receiving approval. This ratio continues to decrease because of the strict regulations required for a new drug to be accepted for use. Thus, to have an economically viable drug discovery and development process, this success rate must be improved.

1.4.1 Single Hit Drugs

Small molecule drug design is an attractive approach broadly pursued by medicinal chemists.[158-162] Single small molecule drugs that are produced by the single-drug-to-single-target approach can manage disease states effectively by acting selectively on a single target molecule. This monotherapeutic approach has succeeded in developing new, safe and effective medicines that are currently on the market. For instance, Tamsulosin **118** (**Figure 1.4**) is a selective α_{1a}-adrenoceptor antagonist single hit drug used to treat the symptoms of benign enlargement of the prostate (BPH).[163] BPH is a disorder prevalent in older men and is characterized by the enlargement of the prostate which often results to symptoms of urinary dysfunction. The development and clinical success of Glivec **119** (STI571, Imatinib) was a highlight for the therapeutic benefit of single molecular targeted therapies.[164,165] Glivec is the first tyrosine kinase-specific inhibitor approved for the treatment of chronic myeloid leukemia - a type of cancer in which the bone marrow makes too many white blood cells.[166]

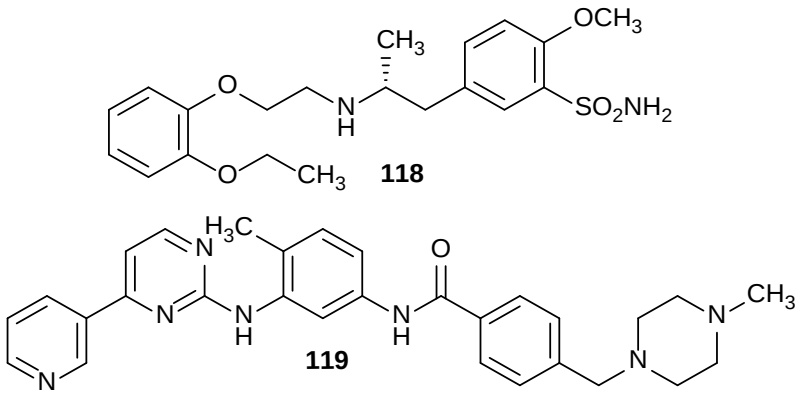


Figure 1.4. Examples of single hit drugs: Tamsulosin **118** and Glivec **119**.

The therapeutic success of single target drugs is however, hampered by their vulnerability to drug resistance.[167-169] Also, many diseases (especially cancer) are complex because they have multiple pathophysiological pathways.[170] The pathways have diverse therapeutic targets which may not be managed effectively by a single-target-drug. Also, single hit drugs are unlikely to manage secondary drug targets associated with a disease state.[171]

1.4.2 Combination Drug Therapy

One approach currently used to overcome the limitations of the 'single hit' drug paradigm is combination drug therapy. The combination drug therapy approach combines two or more known pharmacophores into a single formulation that regulates multiple drug targets at the same time. Combination drugs are the standard of care for complex diseases like cardiovascular diseases,[172] HIV,[173] cancer,[174] and Type 2 diabetes (**Figure 1.5**).[175-177] For example, Actoplus Met (**120 + 121**) is a fixed-dose combination drug approved for the treatment of type 2 diabetes.[176]

Treating patients with combination drugs minimizes the side effects associated with most single hit drug therapies. Despite the benefits of combination drug therapy, there still remain rational objections to it because of setbacks experienced in the formulation process and in the therapeutic outcomes.[178,179] That two separate drugs work on one or more targets in a disease state is not a guarantee that their efficacies will be either retained or increased if they are combined into a single tablet.

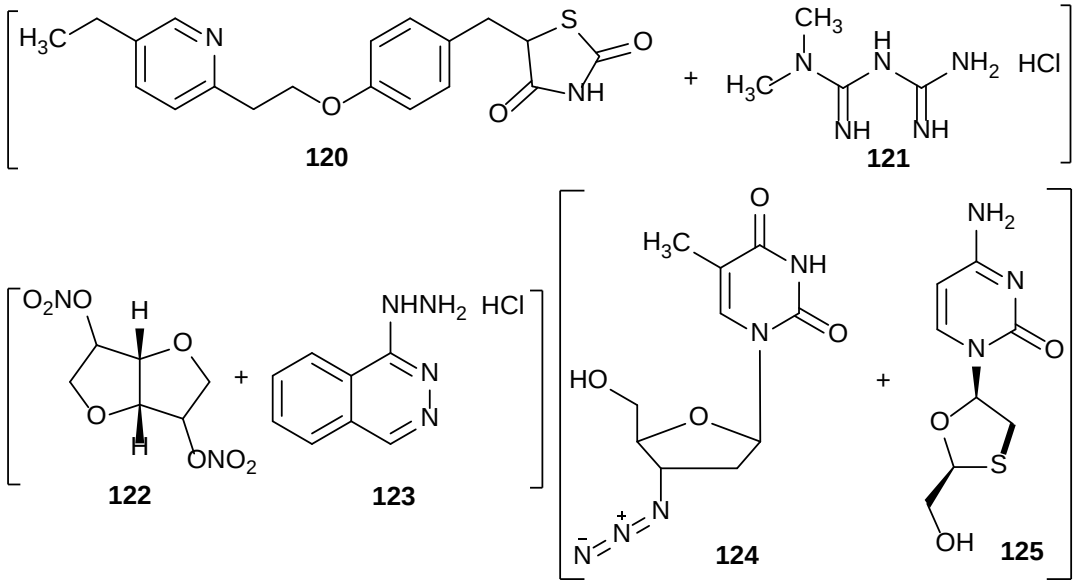


Figure 1.5. Examples of combination drugs: Actoplus Met (pioglitazone **120** + metformin **121**), BiDil (isosorbide dinitrate **122** + hydralazine hydrochloride **123**) and Cambivir (zidovudine **124** + lamivudine **125**).

For instance, combined drugs may interact with each other.[180] Such drug-to-drug interaction can either enhance or reduce the therapeutic efficacy of the combined

drugs. The combined drugs may compete with each other for binding sites in the blood plasma and within target compartments. Thus, the effective therapeutic dose of a low affinity drug may be reduced if administered in combination with a high affinity drug.

The effective dose of combined drugs can also diminish during passive diffusion-dependent processes such as uptake, distribution and elimination. Therefore if two drugs differ significantly in solubility, how each drug gets absorbed and released into the blood stream may affect their effective dose.

Each component of a combination drug (or single hit *cocktail*) has a distinct biological half-life. The half-life of each drug molecule dictates how its steady-state level of accumulation changes within a specific dose interval.[151,181] If over a given dose interval, the rate of elimination of one compound is slower than its uptake rate, then the concentration of that compound will gradually increase over time, which in turn causes an increase in the steady state level of accumulation of that component.

For combination and *multiple* single hit drug therapy, it is difficult to precisely predict the pharmacokinetic and pharmacodynamic properties of an individual drug. The pharmacokinetic and pharmacodynamic properties of a drug provide useful information on how a drug works, its toxicity, and patient compliance.[182]

To address the limitations and difficulties associated with both single hit and combination drugs, Morphy and co-workers[183] proposed a rational drug design approach commonly referred to as pharmacophore hybridization.

1.4.3 Pharmacophore Hybridization

Pharmacophore hybridization (PH), also referred to as molecular hybridization, is a novel drug design concept that represents a promising alternative to both the combination and single hit drug design processes. In PH, functional moieties from two or more pharmacophores are chemically linked to produce a single, non-identical, dual hybrid drug that retains the therapeutic actions of its parent templates.[183-186] Hybrid pharmacophores, like the dual action agents, can modulate one or more drug targets simultaneously. The PH strategy targets bioactive

compounds that are either on the drug market or considered promising drug candidates. Hybrid pharmacophores are less prone to resistance and side effects because of their multi-target action. The pharmacokinetic and pharmacodynamic properties of a hybrid drug are easier to predict than combination drugs. In biological systems, the solubility of a hybrid drug is measured for the whole molecule rather than individual active components. Hence, the relative bioavailability and effective dose of each functional moiety of the hybrid remains undiminished during passive diffusion processes like uptake and distribution. One benefit observed with hybrid drugs is how each component boosts the therapeutic potential of the other.[187,188]

Hybrid pharmacophores are classified based on the how the components are joined together and the degree of integration. The four common ways by which the pharmacophores can be linked together are discussed below.[183,189,190]

1.4.3.1 Cleavable Linker Hybrids

With cleavable hybrids, the functional moieties are linked directly by hydrolyzable bonds, most commonly in the form of esters, amides, carbamates, and disulfides (**Figure 1.6**).[191-195] In biological systems, the individual active component is released by enzymatic cleavage of the linker (e.g. ester bonds cleaved by esterase).

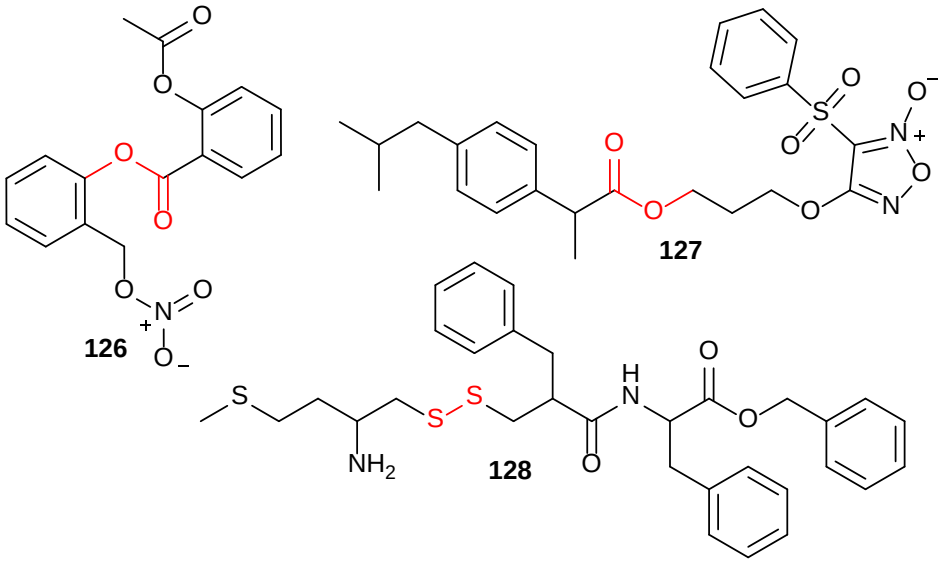


Figure 1.6. Cleavable conjugates: NO-aspirin (NCX4016) **126**, ibuprofen-furoxan NSAID hybrid **127**, and disulfide-based cleavable conjugate RB101 (**128**).

For instance, hybrids of nitric oxide (NO)-releasing agents linked with known anti-inflammatory agents, **126** and **127**, displayed promising synergism by retaining the therapeutic potential of parent templates and at the same time reducing their side effects such as NSAID-induced gastrotoxicity. The disulfide-linked hybrid inhibitor RB101 **128** displays good analgesic properties with longer pharmacological effect.[196]

1.4.3.2 Non-Cleavable Linker Hybrids

Non-cleavable conjugates are linked by a non-hydrolyzable chemical bond or spacer. These agents are more stable and are not generally cleaved by biological enzymes. Notable examples of such conjugates (**Figure 1.7**) work on either the same target, or they may affect two or more closely related targets independently. The platinum–acridine conjugate **129** has a non-cleavable Pt–S linkage that is resistant to nucleophilic attack by the nitrogen atom of a nucleobase.[197,198] This hybrid platinum–acridine compound **129** is a new type of DNA-targeted cytotoxic agent that acts selectively on many cancer cell types - in particular, glioblastoma multiforme (the most aggressive form of metastatic brain tumor).[199] The hybrid phenylindole (2-PI)–aniline mustard **130** consists of two pharmacophore subunits. The phenylindole component is a synthetic ligand for estrogen receptor and the aniline mustard component is a DNA damaging agent. This hybrid **130** is a promising chemotherapeutic agent that is selectively toxic to estrogen receptor-positive breast cancer cells.[189,200]

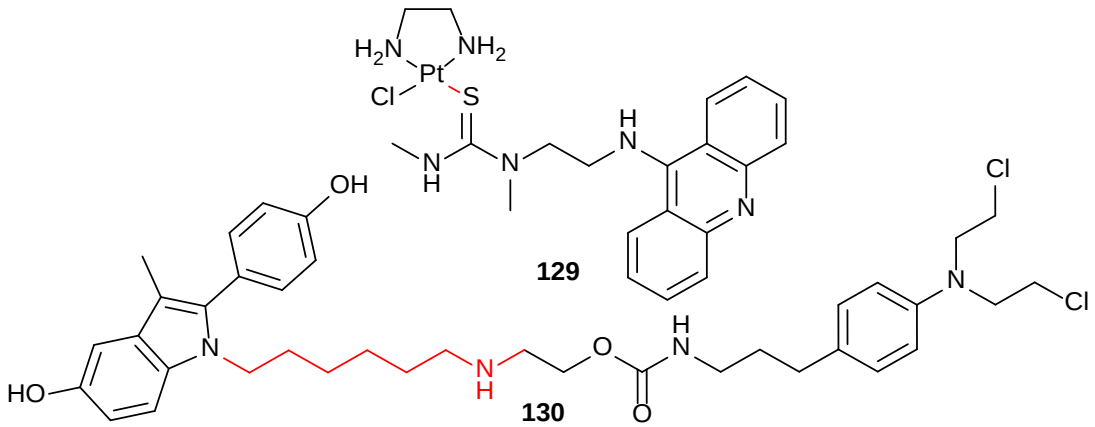
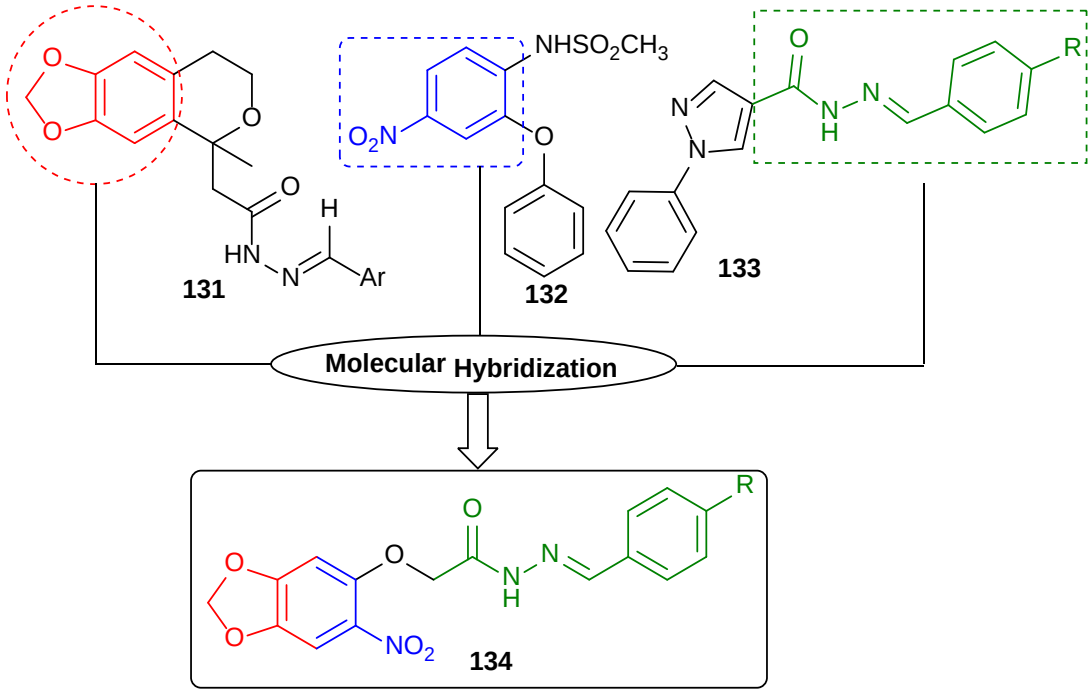


Figure 1.7. Non-cleavable conjugates: PT-ACRAMTU **129** and phenylindole-aniline mustard **130**.

1.4.3.3 Merged and Overlapped Hybrids

The most common type of pharmacophore hybridization involves merging or overlapping structural moieties/elements from two or more pharmacophores. The approach takes advantage of similar structural elements within the parent pharmacophore. Such hybrids range from slightly overlapped to highly integrated systems in which the new hybrid may be completely different in structure to the parent templates. **Scheme 1.34** below depicts an example of how a merged/overlapped pharmacophore hybridization strategy was used to rationally design and synthesize a series of prototype analgesic and antipyretic agents **134** by taking advantage of the structural similarities of the parent analgesic templates (**131-133**).[201,202] This series of hybrid analgesic agents **134** combines the isochromanyl, imesulide and acylarylhydrazone biologically relevant functional subunits of parent compounds **131**, **132**, and **133** respectively.



Scheme 1.34. Synthesis of a series of merged-prototype NSAIDs via pharmacophore hybridization strategy. **R** = H, Br, OH, NO_2, SCH_3, $SOCH_3$, SO_2CH_3, $N(CH_3)_2$.

These examples give insight into how useful and attractive the pharmacophore hybridization strategy is in modern drug design for the development of novel

therapeutics that can modulate complex diseases with multiple-target mechanisms. In most complex disease states such as cancer, arthritis, atherosclerosis and Alzheimer's, the mechanisms underlying such disease states, at the molecular level, are not fully understood. However, both chronic inflammation and oxidative stress (OS) have been established as the major contributors to the multiple pathways through which most cellular damages associated with these complex diseases occur.[138,203,204] Thus, by use of PH strategy, multi-target antioxidant and anti-inflammatory agents are designed to effectively modulate chronic inflammatory and reactive oxygen species-induced cellular damage.

1.5 Project Proposal

Pharmacophore hybridization is an effective and rational drug discovery and development strategy that is currently pursued in both academia and the pharmaceutical industry in the search for new drug candidates. This PhD project uses the pharmacophore hybridization strategy to synthesize new hybrid molecules as potential therapeutics for diseases associated with oxidative stress. The aim was to synthetically combine antioxidant nitroxides with known pharmacophore subunits to produce hybrid agents that may regulate multiple biological targets simultaneously. As oxidative stress, whether drug–induced or from a pathophysiology of a disease state, contributes to the mechanism underlying the pathophysiology of chronic inflammatory diseases and neurodegenerative disorders, the successful fusion of antioxidant nitroxides with parent therapeutics may produce hybrid molecules that minimize oxidative stress and at the same time retain the therapeutic benefits of the parent structure. The non-steroidal anti-inflammatory drugs and the amphetamine-stimulant therapeutics were the main drug classes of interest.

Chapter 2 of this thesis explores the synthesis of overlapped hybrid conjugates that incorporate antioxidant nitroxides with the salicylate class of nonsteroidal anti-inflammatory drugs. Taking advantage of the structural similarities of the parent templates (**Figure 1.8**), **Chapter 2** aims to merge the pyrroline nitroxide **3** with salicylic acid **135** and acetylsalicylic acid **136** (aspirin) to produce new hybrid nitroxide-salicylate molecules 5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2yloxyl **137** (salicylic acid-TMIO) and 5-carboxy-6-acetoxy-1,1,3,3-

tetramethylisoin-2-yloxyl **138** (aspirin-TMIO) shown in **Figure 1.9**. The *o*-acetamide benzoic acid derivative **139** was the ultimate target compound. Compound **139** provides means to compare the biological efficacy of amides to esters. Some preliminary biological data for the antioxidant and anti-inflammatory activities of the target compounds are also discussed.

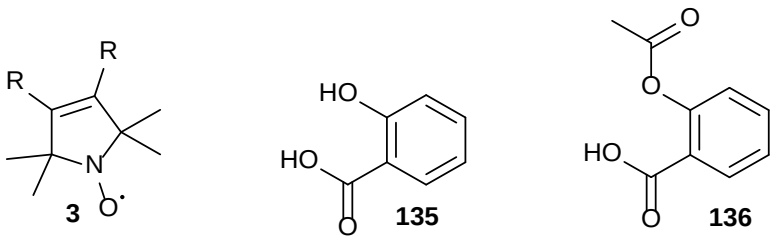


Figure 1.8. Structures of parent molecular templates: pyrroline nitroxide **3**, salicylic acid **135**, and aspirin **136**.

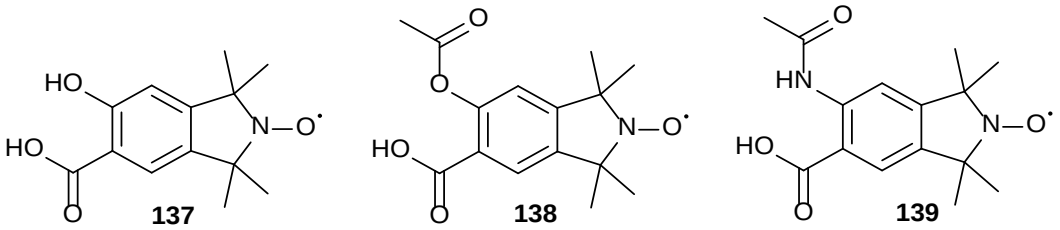


Figure 1.9. Structures of merged-hybrid target molecules: salicylic acid TMIO **137**, aspirin TMIO **138** and *o*-acetamide benzoic acid TMIO **139**.

Chapter 3 probes the synthesis of cleavable and non-cleavable hybrid nitroxide conjugates of non-steroidal anti-inflammatory drugs, mainly salicylates and indomethacin. The nitroxide moieties were linked to the parent drugs through ester, amide and amine linkages. To obtain more structural activity relationship data, the nitroxides were tethered to the parent pharmacophores at various positions (**Figure 1.10**). The common cyclic nitroxide ring classes (pyrroline, piperidine and isoindolines) were used in the synthesis. As steric bulk around the nitroxide moiety influences the rate of biological reduction, the synthesis of tetramethyl and tetraethyl conjugates was also undertaken. To compare the antioxidant potential of the various NSAID-nitroxide conjugates, the conjugates of some common antioxidants (Trolox and Cinnamic acid) were also synthesized (**145** and **146**). Preliminary biological data for some selected target compounds is also discussed.

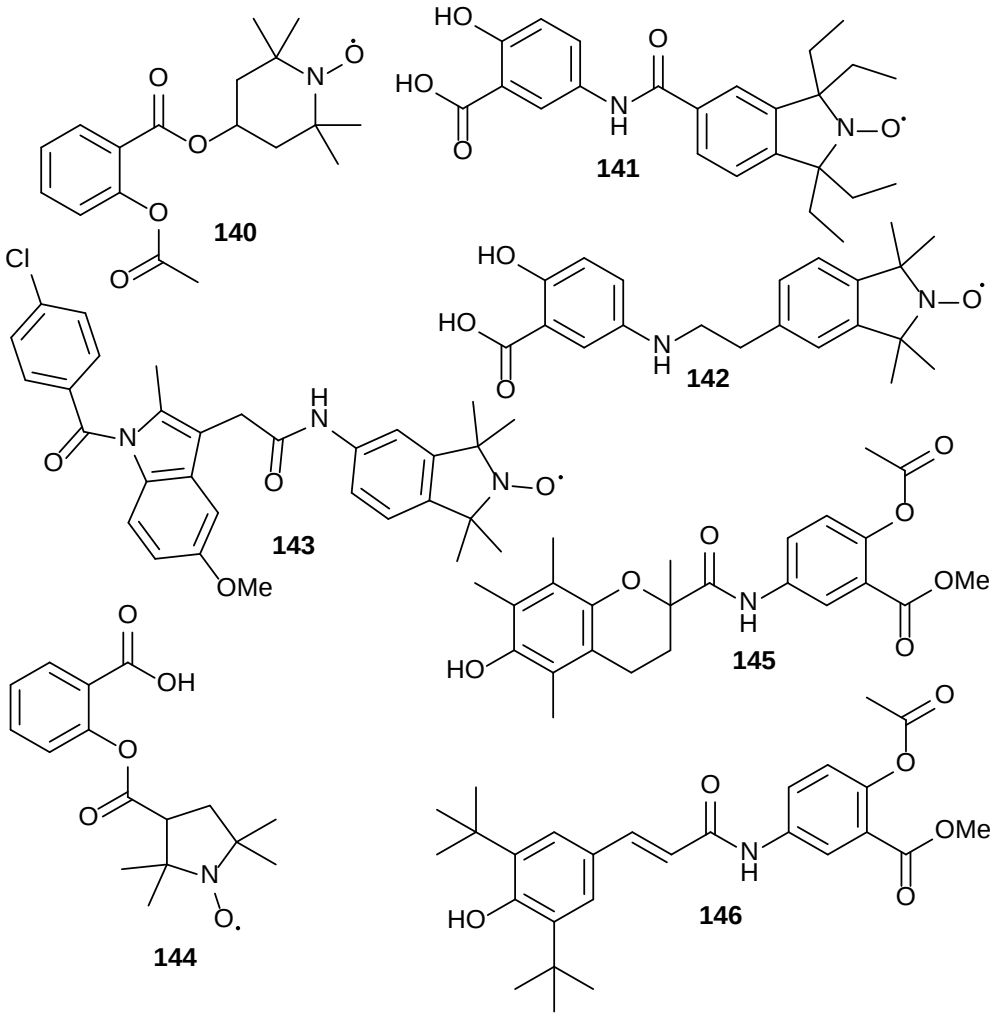


Figure 1.10. Representative targets of cleavable and non-cleavable NSAID-nitroxide conjugates.

Chapter 4 focuses on the enantioselective synthesis of overlapped nitroxide hybrids of the sympathomimetic amine stimulants L-Dopa **147** and Ritalin **148 (Figure 1.11**). L-Dopa and Ritalin are the main therapeutic agents used to treat Parkinson's disease and attention deficit/hyperactivity disorder (ADHD) respectively.

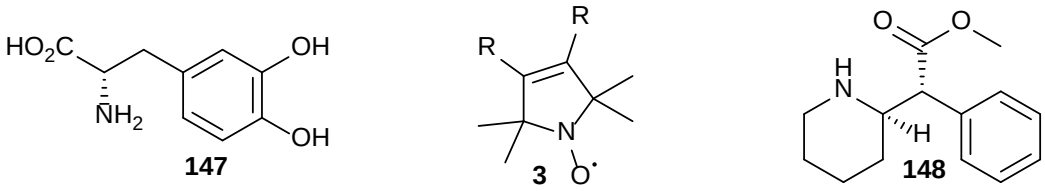


Figure 1.11. Parent templates: L-Dopa **147**, pyrroline nitroxide **3** and Ritalin **148**.

As oxidative stress contributes to the pathophysiology of Parkinson's disease and ADHD, the target hybrid nitroxide molecules **149** and **150** (**Figure 1.12**) could serve as effective therapeutic agents that reduce oxidative stress by the antioxidant action of the nitroxide moiety and at the same time retain the therapeutic benefits of the parent structure.

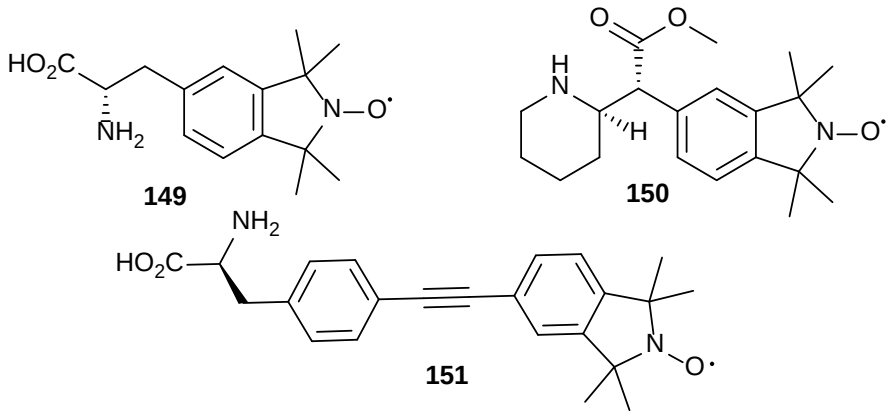


Figure 1.12. Chemical structures of target compounds: merged psychostimulant-nitroxide target conjugates (L-Dopa TMIO **149** and Ritalin TMIO **150**) and the rigid alkyne-linked chiral α-amino acid nitroxide spin label **151**.

The rigid alkyne-linked chiral α-amino acid nitroxide **151** is the final target compound of **Chapter 4**. Unlike the L-Dopa and Ritalin nitroxide analogues (**149** and **150**), compound **151** is designed as a site directed spin label for studying the structure and structure and conformational dynamics of peptides and proteins. The preparation of **151** will be carried out by taking advantage of similar asymmetric α-amino acid synthetic approach that will be employed for the synthesis of the L-Dopa nitroxide analogue **159**. Due to its rigidness, chirality and enantiopurity, compound **151**, if incorporated into a peptide, could provide relevant structural and dynamic information by EPR spectroscopy without any drawback associated with rotational mobility, isomerization, multiple conformers, and achiral effects.

Chapter 5 presents the synthesis of the novel isoindoline catechol and benzodioxole amines, and their corresponding nitroxides. The target compounds are designed for potential use in biology as antioxidants but they could also have applications in material science as building blocks for nitroxide-functional materials. The

tetramethyl and tetraethyl substituted isoindoline derivatives **154-161** of each parent template were the target compounds (**Figure 1.14**).

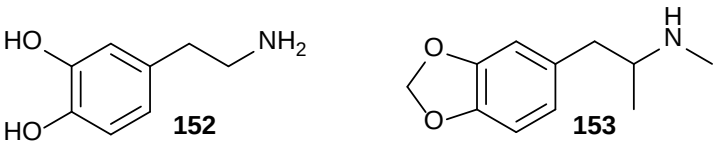


Figure 1.13. Parent pharmacophores: dopamine **152** and 3,4-methylenedioxy-methamphetamine **153** (MDMA/Ecstasy).

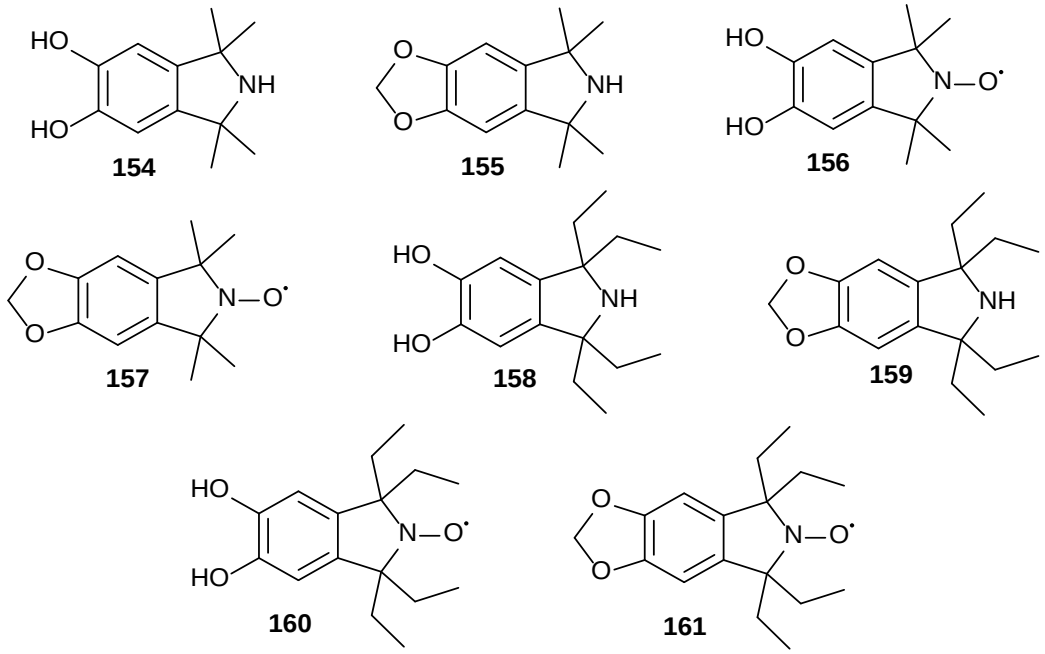


Figure 1.14. Target compounds: Catecholamines tetramethylisoindoline (TMI) **154** and tetraethylisoindoline (TEI) **158**; catechol TMIO **156** and TEIO (tetraethylisoindolin-2-yloxyl) **160**, benzodioxole TMI **155** and TEI **159**; benzodioxole TMIO **157** and TEIO **161**.

Chapter 6 gives a summary of the project and recommendations for future work.

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Chapter 2: Merged Dual-Action Nitroxide-Aspirin Hybrids

2.1 Background: Cellular Inflammation- Friend or Foe?

Cells in our body are under constant attack from invading pathogens. Changes occurring at the molecular level that arise from such attacks can lead to cell and tissue damage or the death of the cell.[1-4] However, the immune system, comprising innate and acquired defences, limits the harmful effects of pathogens and injuries.[5] Regardless of the stimuli, innate cellular defence systems rapidly respond to infections and tissue damage through a complex cascade of events aimed at restoring physiological homeostasis. An integral component of these defence systems is the cellular inflammatory response. Primarily triggered by tissue damage (caused by trauma or infection), controlled-cellular inflammatory response (acute inflammation) facilitates pathogen clearance, tissue recovery and regeneration processes at infected or damaged sites.[5] The response is characterized by the action of a diverse range of inflammatory mediators (such as prostaglandins (PGs), leukotrienes, bradykinin, platelet-activating factor, cytokines and interleukin-1 and several more broadly acting processes such as vasodilation and coagulation.[6,7] Coagulation, for example, minimizes the loss of blood from damaged vessels by forming platelet aggregates at the injured sites. While neutrophils initiate pathogen killing by phagolysosomes, macrophages on the other hand, clear all matrix and cell debris and release inflammatory mediators that act as regulatory tissue-repair signalling molecules.[8] However, when an acute inflammatory response is not properly regulated, it can transform into a chronic inflammatory response. Chronic inflammation is mainly characterized by oxidative stress and prolonged and excessive inflammation which may translate into an irreparable damage to host tissues and, in severe situations, organ malfunction or death.[9,10] Chronic inflammation is a significant contributing factor to numerous pathophysiological conditions including rheumatoid arthritis, cancer, neurodegeneration and cardiovascular diseases. [7,8,11-18]

Rheumatoid arthritis, is an autoimmune disease that afflicts about 1% of the world's population.[19] The symptoms and mechanism of progression of rheumatoid arthritis are mainly characterized by chronic inflammation of the synovial joints. This results in lingering pain, loss of function and disability. In general, nonsteroidal anti-inflammatory drugs (NSAIDs) are the most common therapeutic agents used to manage inflammatory symptoms.[17,20-22] NSAIDs are a structurally diverse group of drugs (**Figure 2.1**) each with a similar mode of action.

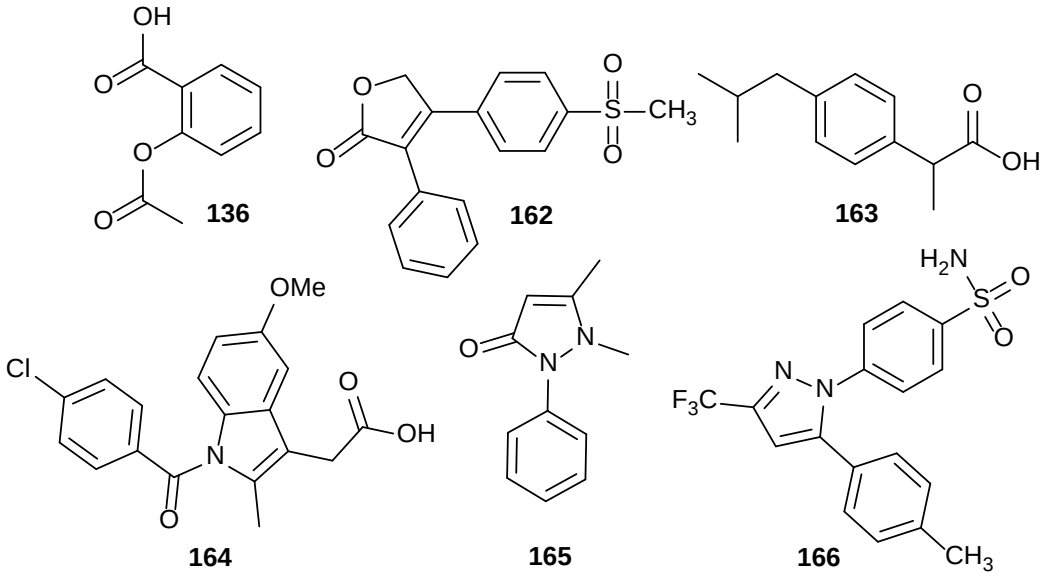


Figure 2.1. Common NSAIDs: aspirin **136**, Vioxx **162**, ibuprofen **163**, indomethacin **164**, antipyrine **165** and celecoxib **166**.

NSAIDs exert their therapeutic action mainly as antipyretics (drugs used to manage fever), anti-inflammatories and analgesics (pain relief medications), by inhibiting the activity of several enzymes and ion channels.[3,6,7,21-29] The main target enzyme for NSAIDs is the *cyclooxygenase* (COX) enzyme. COX is a membrane-bound hemoprotein and glycoprotein present primarily in the endoplasmic reticulum of prostanoid-forming cells.[6,7] It has two main isoforms (COX-1 and COX-2) which have similar active sites, but differ slightly in size. COX-1 is the constitutionally expressed isoform present in almost every tissue. Under physiological conditions, COX-1 catalyzes the production of prostaglandins (PGs) involved in basic cytoprotective functions such as maintaining the gastrointestinal (GI) mucosal integrity, vascular homeostasis, and renal regulation. COX-2 is the inducible

expressed isoform involved in producing PGs mainly in response to inflammatory stimuli from infections or injuries.[7,17,20] The problem with most traditional NSAIDs such as indomethacin and aspirin is they inhibit both COX-1 and COX-2 enzymes (**Figure 2.2**).[30,31]

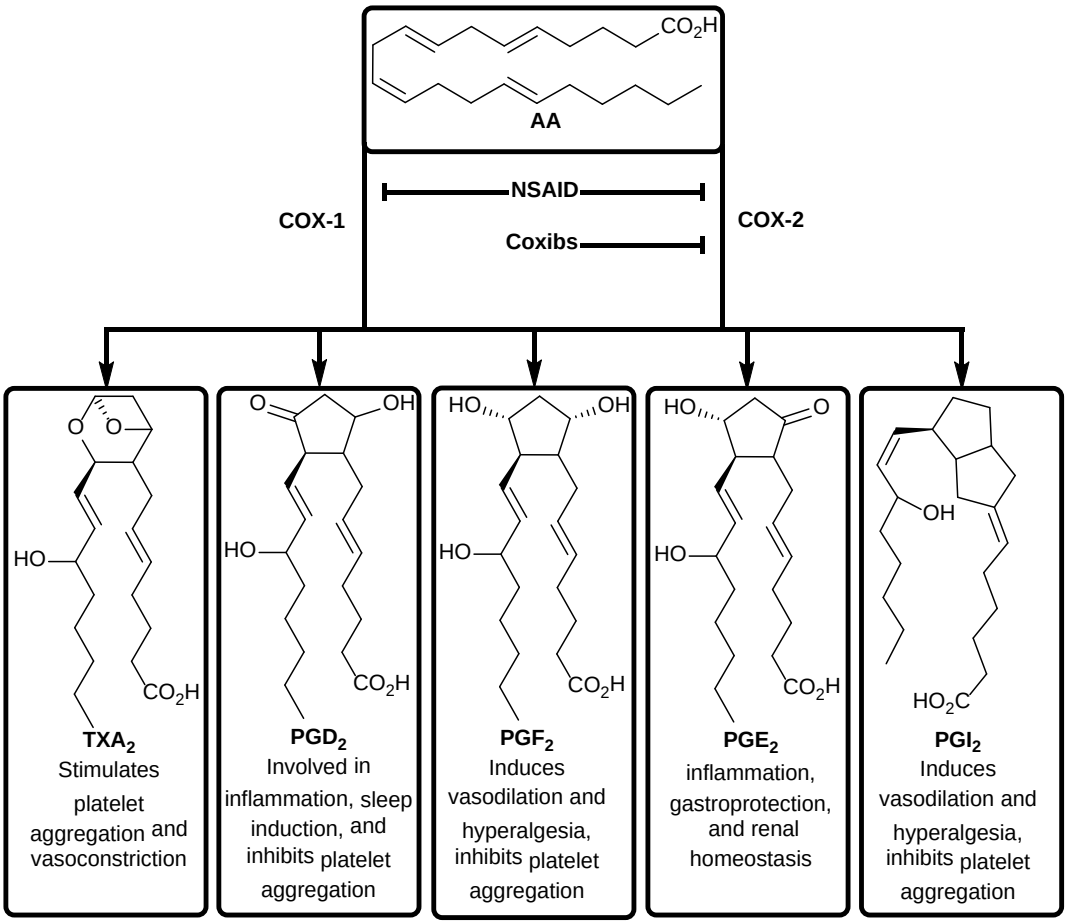


Figure 2.2. COX-catalyzed biosynthesis of prostanoids and their roles in inflammation and normal cell physiology: COX catalyzes the metabolism of arachidonic acid (**AA**, released from phospholipid bilayer) to various prostanoids including thromboxane A (**TXA_2**), prostaglandin D_2 (**PGD_2**), prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**), prostaglandin E_2 (**PGE_2**) and prostacyclin (**PGI_2**)- that are involved in numerous cellular and inflammatory processes. Adapted and modified from Cipollone *et al*.[30]

Such inevitable nonselectivity by conventional NSAID therapy leads to adverse side effects- notably gastrointestinal ulceration and bleeding, platelet dysfunction and renal complications, as a result of the limited number of required cytoprotective

prostaglandins.[32] Logically, selective COX-2 inhibitors (Coxibs) were developed as effective inflammatory agents without the adverse side effects normally associated with chronic use of conventional NSAIDs.[33] Celecoxib (marketed by Pfizer under the brand name Celebrex) and rofecoxib (Vioxx) are two licensed selective COX-2 inhibitors that have been marketed for the treatment of acute pain, osteoarthritis and rheumatoid arthritis.[34,35] However, the use of most COX-2 selective inhibitors has been hampered by the increased risks of cardiovascular disorders observed in patients.[36-38] As a result, some selective COX-2 inhibitors have been removed from the drug market following legal action.[39]

As described previously, oxidative stress is a major contributor to inflammatory diseases.[40-42] Also, endoscopic studies have shown that elevated levels of reactive oxygen species (ROS) are a key factor in both the pathogenesis and the propagation mechanism underlying NSAID-induced gastric mucosa ulceration.[43] The abnormal level of ROS produced during NSAID-induced ulceration is linked to the increase in the activity of the myeloperoxidase enzyme. During NSAID-induced ulceration, gastrointestinal endothelial cells also show significant suppression (to below physiological levels) of endogenous antioxidant systems such as superoxide dismutase (SOD) and glutathione. Such high levels of ROS cause mucosa damage either directly or via ROS-induced lipid peroxidation. This in turn leads to increased production of ROS oxidants. Thus, to effectively manage chronic inflammatory diseases and the associated NSAID-induced damage, there is a clear need for both an effective anti-inflammatory and an antioxidant intervention. This project explores the design of novel, potential dual-action based pharmacophores that contain both anti-inflammatory and antioxidant functional subunits. Using the pharmacophore hybridization strategy, the aim was to combine antioxidant nitroxides with the salicylate type NSAIDs by taking advantage of structural similarities of the parent compounds. Specifically, drug candidates were designed whereby a pyrroline nitroxide **3** was merged with salicylic acid **135** and aspirin **136** pharmacophores (**Figure 2.3**) to produce new hybrid nitroxide-salicylate molecules 5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **137** (salicylic acid TMIO) and 6-acetoxy-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **138** (aspirin TMIO) shown in **Figure 2.4** below. In addition, the *o*-acetamide benzoic acid derivative **139** was

also conceived as a target as it differs structurally from aspirin TMIO **138** by having an *ortho*-acetamide group instead of an *ortho*-acetate. This variation has the potential to provide structural-reactivity relationship information that could provide insight on the process and mechanisms of competitive inhibition of the COX enzyme by the compounds. Also, the mechanism of action of aspirin is thought to involve an irreversible acetylation of an amino acid (serine 530) of the COX active site.[44]

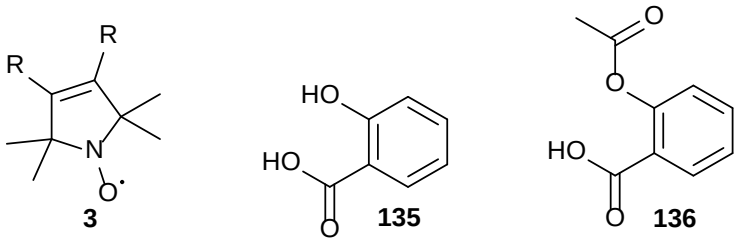


Figure 2.3. Structures of parent molecular templates: pyrroline nitroxide **3**, salicylic acid **135**, and aspirin **136**.

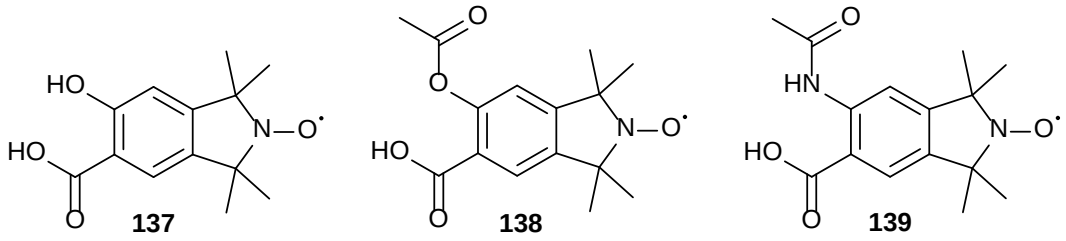


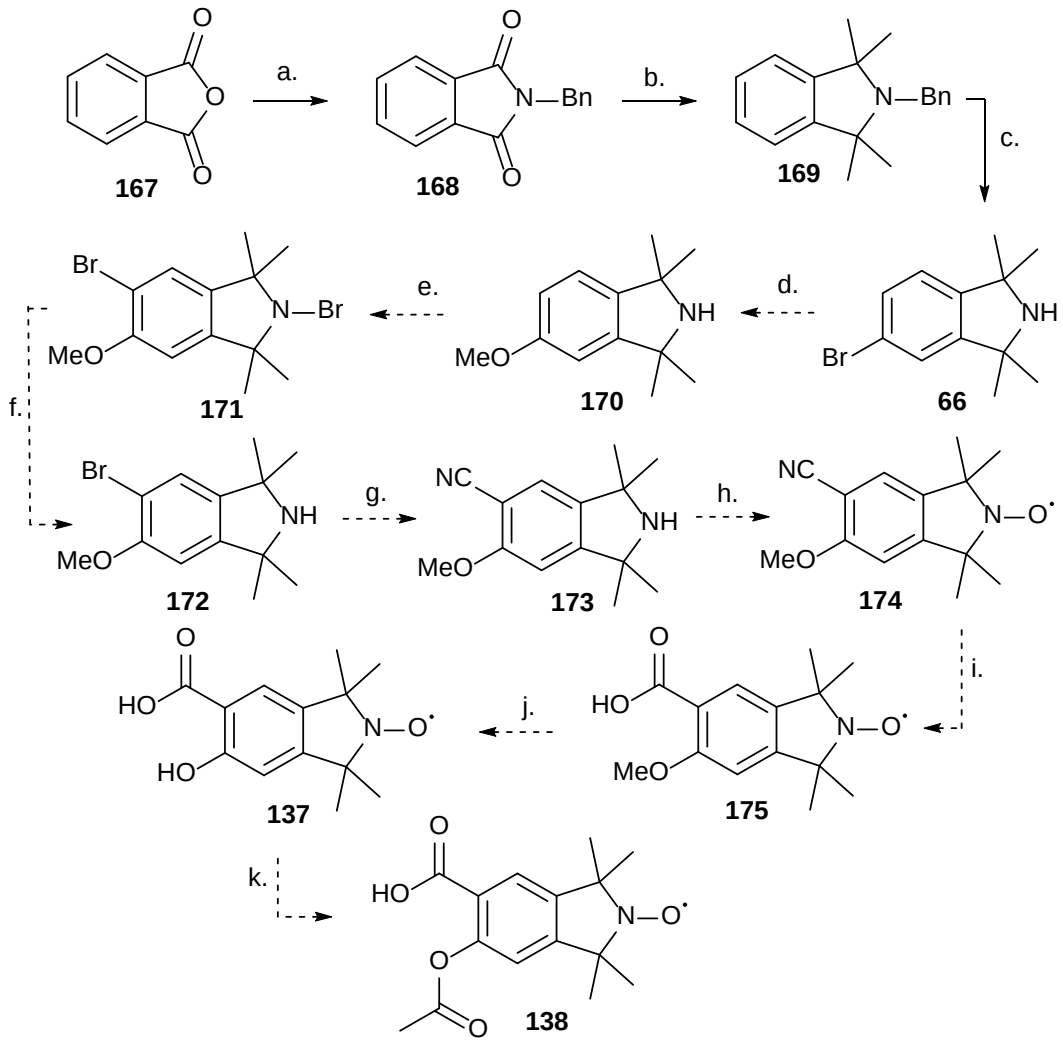
Figure 2.4. Structures of target hybrid molecules: salicylic acid TMIO **137**, aspirin TMIO **138** and *o*-acetamide benzoic acid TMIO **139**.

Such transesterification of the acetate (present in target compound **138**) would not be possible for the target amide nitroxide **139**. Therefore any efficacy of the structurally similar acetamide **139** would arise purely from either the nitroxide moiety or by a time-dependent reversible hydrogen bonding, ionic, van der Waals or hydrophobic interactions with distinct amino acid residues of the COX.[45] The synthesis and preliminary biological results of these novel compounds are discussed in the next section.

2.2 Results and Discussion

2.2.1 Synthesis of Salicylic Acid and Aspirin TMIO

Scheme 2.1 below outlines the synthetic route that was proposed for the synthesis of salicylic acid and aspirin TMIO conjugates **137** and **139**. Dashed arrows represent new work first described in this thesis.

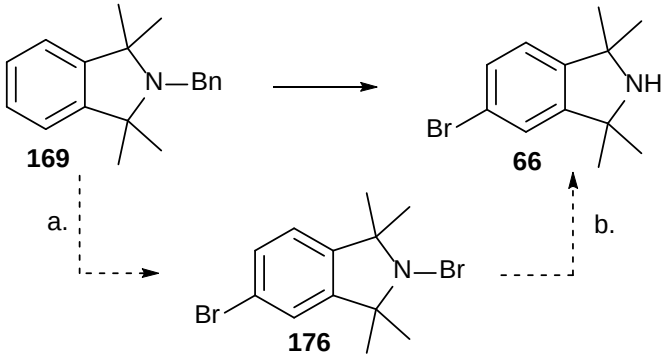


Scheme 2.1. Overall route for the synthesis of salicylic acid TMIO **137** and aspirin TMIO **138**. Reagents and conditions: a. $BnNH_2$, AcOH, reflux, 1 h, 98%; b. MeMgI, Et_2O, toluene, reflux, 3 h, 27%; c. Br_2, $AlCl_3$, DCM, then $NaHCO_3$, H_2O_2, MeOH, 1h, 90%; d. NaOMe, DMF, MeOH, reflux; e. Br_2, $AlCl_3$, DCM; f. H_2O_2, MeOH; g. $K_4[Fe(CN)_6]$, *n*Bulmi, CuI, toluene; h. *m*CPBA, DCM; i. NaOH, H_2O reflux; j. BBr_3, DCM; k. AcCl, TEA, THF.

2.2.1.1. Synthesis of 5-Bromo-1,1,3,3-tetramethylisoindoline 66

The 5-bromo-1,1,3,3-tetramethylisoindoline **66** precursor was synthesized in three steps from phthalic anhydride **167** by following previous established literature procedures.[46] Thus, commercially available phthalic anhydride **167** was reacted with excess benzylamine and the resulting reaction mixture was refluxed in acetic acid to give a crude residue that was recrystallized from ethanol to afford *N*-benzylphthalimide **168** as white fluffy crystals in 98% yield. The melting range (116-117 ^{o}C) was consistent with that reported in the literature.[47] The synthesis of *N*-benzyl-1,1,3,3-tetramethylisoindoline **169** was then performed using freshly prepared methyl magnesium iodide. In this case, *N*-benzylphthalimide **168** was tetramethylated by reacting with six equivalents of freshly prepared methyl Grignard reagent in refluxing toluene. The crude product residue obtained was purified by filtering through Celite and a short silica gel column, followed by recrystallization from methanol to give *N*-benzyl-1,1,3,3-tetramethylisoindoline **169** as a white crystalline solid in a typical modest yield of 27%. The low yield was attributed to the formation of a large amount of intractable purple material that was separated from **169** at the basic alumina filtration step.[48-50] All physical data obtained for compound **169** were in agreement with that previous reported in the literature.[51]

To synthesize bromoamine precursor **66** from compound **169**, a combined oxidative debenzylation and selective ring mono-bromination reaction was carried out (**Scheme 2.2**).

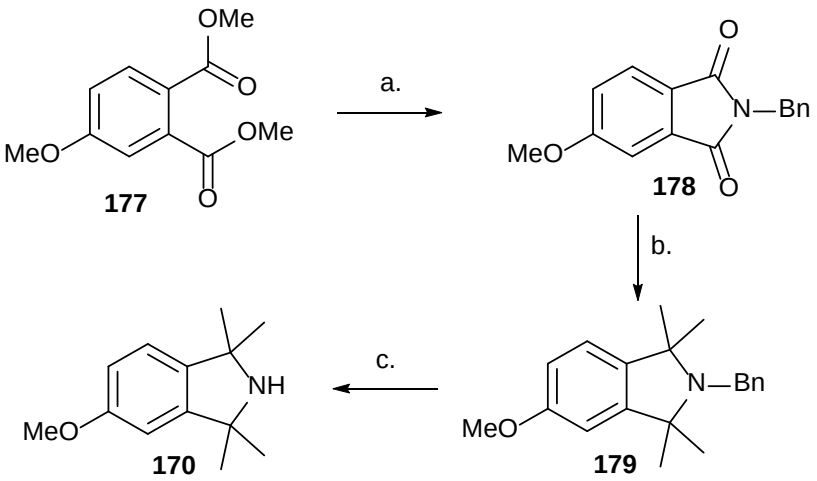


Scheme 2.2. Synthesis of bromoamine precursor **66**. Reagents and conditions: a. Br_2, $AlCl_3$, DCM, 0 ^{o}C, 1 h; b. $NaHCO_3$, H_2O_2, MeOH, (90% over the two steps).

When compound **169** was treated with an almost stoichiometric amount of bromine (2.1 equiv.) in the presence of anhydrous aluminium trichloride, the crude product obtained, predominantly as the 2,5-dibromo **176** intermediate, was then reduced to the desired bromoamine **66** using hydrogen peroxide in the presence of sodium bicarbonate. Pure bromoamine **66** was obtained as a pale white solid in 90% yield with all characteristic data obtained matching those reported in the literature.[46]

2.2.1.2. Copper-Catalyzed Methanolysis of Bromoamine 66

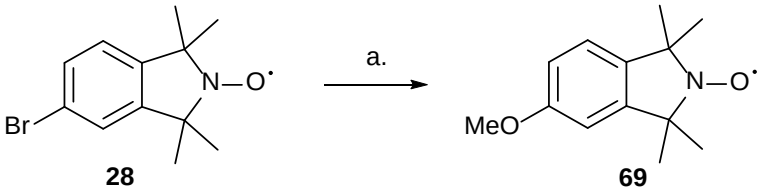
The only literature procedure to describe the synthesis of the 5-methoxyamine intermediate **170** was reported in a patent by Haj-Yehia and coworkers.[52] In their work, compound **170** was prepared in three steps from dimethyl 4-methoxyphthalate **177** (**Scheme 2.3**). Although it is commercially available, compound **177** is expensive when compared to other phthalate precursors such as the phthalic anhydride **167**. In addition, no yields were reported for this approach.



Scheme 2.3. Synthesis of 5-methoxyamine **170** from 4-methoxyphthalate **177**. Reagents and conditions: a. $BnNH_2$, PhMe, reflux; 111°C; b. MeMgI, Et_2O, RT, then PhMe 4-6 h, 111°C; c. H_2, Pd/C, EtOH, 15 h, RT, 50 psi: (no yields reported).[52]

However, the nitroxide derivative of **170** was previously synthesized by Benjamin Morrow of the Bottle research group here at QUT (**Scheme 2.4**).[53] In this case, the 5-bromo nitroxide **28** was subjected to a copper (I) catalyzed methanolysis reaction developed by Capdevielle and Maumy.[54] The method involves refluxing aryl halides

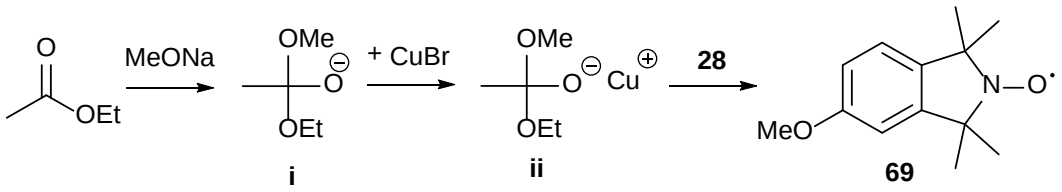
in 5 M sodium methoxide solution in the presence of catalytic amount of copper (I) bromide and ethyl acetate as a co-catalyst.



28 **69**

Scheme 2.4. Copper-catalyzed methanolysis of bromonitroxide **28**. Reagents and conditions: a. NaOMe, CuI, EtOAc, MeOH, reflux, 16 h, 88%.[53]

Since most transition metal catalyzed methoxylations (nucleophilic aromatic substitution with aryl halides) have drawbacks such as difficulties in initiation and low conversion,[55-57] ethyl acetate is often used as an effective co-catalyst to enhance the methanolysis reaction particularly for deactivated aryl halide substrates. Ethyl acetate stabilizes and increases the solubility of the cuprous salts by first forming a stable tetrahedral complex (**i**) with the methoxide ion (**Scheme 2.5**).



i **ii** **69**

Scheme 2.5. Proposed mechanism for the copper-catalyzed methoxylation of **28**.[54]

The tetrahedral intermediate (**i**) is a strong ligand that effectively binds to the Cu(I)-catalyst to generate Cu(I)-complex (**ii**) which assists the substitution process. If no co-catalyst is used, an insoluble yellow copper (I) methoxide is usually formed.[54] Such salts (Cu(I)-methoxide) disproportionate quickly to form some Cu^0 and Cu^{2+} salts. This in turn slows down the catalytic cycle and eventually stops it.

The copper-catalyzed methanolysis method was employed herein for the synthesis of the methoxyamine **170**. However, when bromoamine **66** was refluxed under similar methanolysis conditions, less than 10% conversion of **66** to **170** was observed by ^{1}H NMR spectroscopic analysis. The conversion could not be improved by prolonged reaction (from 1 to 3 days), increased catalyst loading, or the use of copper (I) iodide

in place of copper (I) bromide (**Table 2.1**). In a mechanistic study of the copper (I)-catalyzed methanolysis of aryl halides, Aalten and co-workers used *N*,*N*-dimethylformamide (DMF) as a suitable co-catalyst (or co-solvent) to quantitatively obtain aryl methyl ethers from the corresponding aryl bromides.[58] Like ethyl acetate, DMF stabilizes and increases the solubility of the cuprous-catalyst by forming a Cu-DMF complex.[59,60]

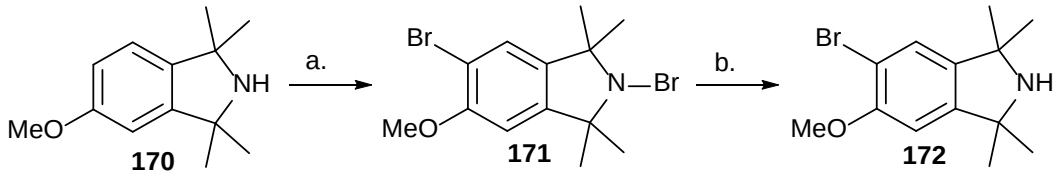
Table 2.1. Optimizing the copper-catalyzed methanolysis of bromoamine **66**.

Catalyst	Catalyst (%mol)	Co-catalyst	Conversion (%)
CuBr	10/20	-	-
CuI	10/20	-	<5
CuBr	10/20	EtOAc	-
CuI	10/20	EtOAc	<10
CuBr	10/20	DMF	<5
CuI	12	DMF	100

However, in this case no conversion was observed when bromoamine **66** was refluxed in sodium methoxide in the presence of DMF and copper (I) bromide. When copper (I) iodide was used instead of copper (I) bromide however, the methanolysis proceeded smoothly with complete conversion of **66** to **170** achieved after refluxing overnight. This is thought to be because of the higher reactivity of the copper (I) iodide with DMF as co-solvent. The crude product obtained was determined to be pure enough by NMR spectroscopy to be used in the subsequent step. It could be further purified if necessary by silica gel column chromatography to afford compound **170** in 88% yield. The purity of **170** was assessed by HPLC analysis to be >95%. Pure compound **170** could also be obtained after acid/base washes of the crude product. The most diagnostic feature was the characteristic methoxy protons (OMe) signal at 3.87 ppm in the ^{1}H NMR spectrum of **170**. Other characteristic data obtained for **170** included ^{13}C NMR, FTIR, and HRMS spectra (see experimental section for details).

2.2.1.3. Synthesis of 5-Bromo-6-methoxy-1,1,3,3-tetramethylisoindoline 172

The next step developed in this synthetic approach involved the selective bromination of **170** at the 6-position to give the bromo ether **172**. This was achieved using similar bromination conditions as were employed for the synthesis of compound **66** (**Scheme 2.2**). Thus, when compound **170** was treated with 2.5 molar equivalents of bromine in the presence of anhydrous aluminium trichloride, a crude fraction of 2,5-dibromo compound **171** was obtained as a light brown oil after extraction and evaporation of the organic solvent (**Scheme 2.6**).



170 **171** **172**

Scheme 2.6. Synthesis of bromomethoxyamine **172** via selective bromination of methoxyamine **170**. Reagents and conditions: a. Br_2, $AlCl_3$, DCM, 0 ^oC, 1 h, 87%; b. $NaHCO_3$, H_2O_2, MeOH, 98%.

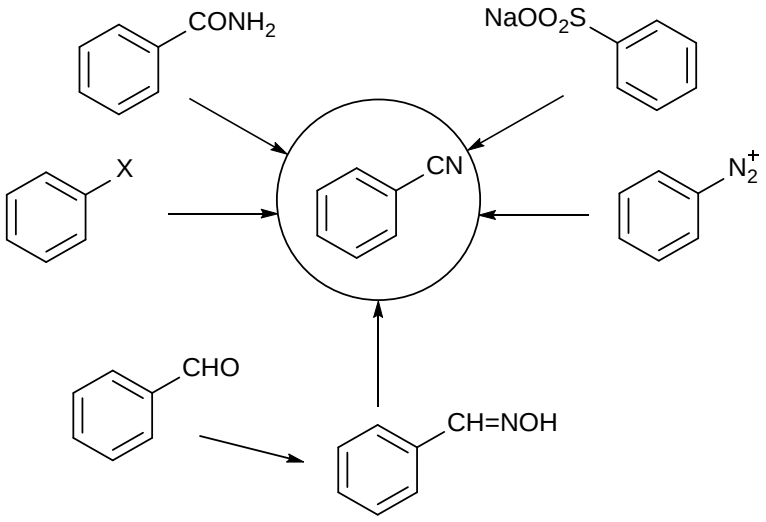
Though the Lewis acid catalyst, aluminium trichloride would typically be expected to be required to brominate the aromatic ring, the *N*-bromination proceeds readily even in the absence of the catalyst. The crude residue was triturated with methanol to give **171** as a yellow solid in 87% yield. Despite the presence of the activating methoxy group, only mono bromination was observed. This is presumably because of the steric hindrance at the 1 and 3-positions. The structure of compound **171** was confirmed by ^1H and ^{13}C NMR and FTIR spectroscopy. In the ^1H NMR spectrum of **171**, two singlets were observed for the two aryl protons at 6.66 and 7.30 ppm. Compound **171** was >93% pure by HPLC and had a melting range of 97-98 ^oC.

It was then determined that compound **171** could be subsequently reduced to the secondary amine derivative, 5-bromo-6-methoxy-1,1,3,3-tetramethylisoindoline **172**, by aqueous hydrogen peroxide in the presence of sodium bicarbonate. A weak N-H stretching frequency was observed in the FTIR spectrum of **172** at 3307 cm^{-1}. The HRMS spectrum of **172** showed the characteristic isotopic pattern for the bromine

atom with the molecular ion plus proton $[M + H]^+$ mass observed for both isotopes at 284 and 286. Although hydrogen peroxide was used to reduce **171** to the secondary amine derivative **172**, the reduction (without hydrogen peroxide) was observed to occur slowly even after **171** was left in a sealed vessel of methanol for 3-7 days.

2.2.1.4. Synthesis of 5-Cyano-6-methoxy-1,1,3,3-tetramethylisoindoline 173 via Copper-Catalyzed Cyanation

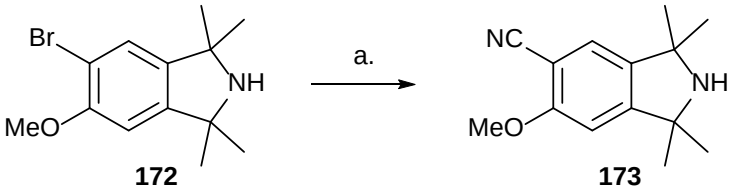
Typically, aryl nitriles are synthesized from five general precursors (**Scheme 2.7**).[61] Choosing a route to a particular aryl nitrile mainly depends on the precursor available, how stable other functional groups are to the chosen reaction conditions, and the prospective overall yield of the route.



Scheme 2.7. Common precursors to aryl nitriles.

The use of aryl halides to synthesize aryl nitriles is an attractive methodology because aryl halides are versatile and readily available stable precursors. The Rosenmund-von Braun reaction of aryl halides with CuCN is the conventional method used to synthesize benzonitriles.[57,62] The drawback to this reaction is that it is relatively less reactive to aryl chlorides and bromides and requires the use of excess CuCN under somewhat demanding reaction conditions. To circumvent these limitations, it was decided to exploit a copper-catalyzed cyanation of aryl bromides as reported by Schareina *et al.*[63] In this case the cyanation of 5-bromo-6-methoxy-1,1,3,3-tetramethylisoindoline **172** was achieved using potassium

hexacyanoferrate(II), as the cyanide source, in the presence of a catalytic amount of CuI with *N*-butylimidazole as a co-solvent (**Scheme 2.8**).



172 **173**

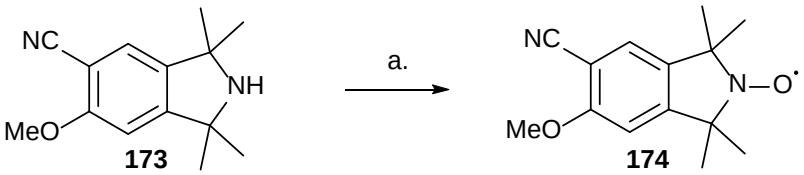
Scheme 2.8. Copper-catalyzed cyanation of bromomethoxyamine **172**. Reagents and conditions: a. $K_4[Fe(CN)_6]$, *n*Bulmi, CuI, *o*-xylene, 180 ^oC, 3 d, 78%.

Unlike most transition metal-catalyzed cyanation methods which use more toxic cyanide sources (KCN, NaCN, $Zn(CN)_2$, and TMSCN),[61,64-68] potassium hexacyanoferrate (II) is the least toxic cyanide source available and is relatively inexpensive. In addition, the Schareina method is tolerant to many functional groups and gives excellent yields with both electron deficient and electron rich substrates. Also, with most other metal-catalyzed aryl halide cyanations, the cyanide sources usually form stable complexes with the metal centre. Such complexes are known to deactivate the catalytic process and result in an inefficient cyanation. Using *N*-butylimidazole can prevent the formation of such undesirable cyano-metal complexes. The *N*-butylimidazole acts as a superior ligand that effectively binds to the metal centre to generate an active cyanation catalyst that is stable enough and selective towards the targeted substrates. When compound **172** was however, refluxed for three days under these conditions at 150 ^oC in toluene, the crude residue obtained was at best a 1:1 mixture of the desired product **173** and the unreacted starting material **172** (determined by ^1H NMR spectroscopy). Attempts to separate these compounds by conventional separation techniques (column chromatography and recrystallization) were unsuccessful. The ratio could not be improved even with increased molar equivalents of reagents and prolonged reaction time (1 week). However, complete conversion was achieved after three days when *o*-xylene was used in place of toluene as solvent. This allowed for the reaction temperature to be raised to 180 ^oC. Using this solvent, compound **173** was obtained as a white solid in 78% yield after purification by column chromatography, followed by recrystallization to remove (presumably) the green Cu-*N*-butylimidazole complex.

Though the residual Cu-*N*-butylimidazole-complex was removed by recrystallization (or sometimes by trituration), its presence did not affect the next reaction and could be readily removed in the workup of the reaction by acid extraction. The FTIR spectrum of **173** showed a distinct sharp and strong C≡N vibration stretch at 2221 cm^{-1}. In addition, the extra (1 more carbon than **172**) carbon signal at 100.8 ppm in the ^{13}C NMR spectrum of **173** supported the presence of a nitrile group. The expected molecular ion plus proton $[M + H]^+$ was observed at m/z 231 in the HRMS spectrum of **173**. As expected, the characteristic isotopic pattern of a single bromine atom (of the starting material **172**) was not observed. The product had a melting range 138-139 ^oC and its purity was assessed by HPLC to be >99%.

2.2.1.5. Synthesis of 5-Cyano-6-methoxy-1,1,3,3-tetramethylisoindolin-yloxyl 174

After the successful synthesis and characterization of the aminonitrile **173**, the next step was to oxidize **173** to the corresponding nitrile nitroxide **174**. This can be achieved using *m*CPBA or hydrogen peroxide oxidation conditions. Thus, compound **173** was converted to the nitroxide derivative **174** by reacting with 1.2 molar equivalents of *m*CPBA in dichloromethane (**Scheme 2.9**).



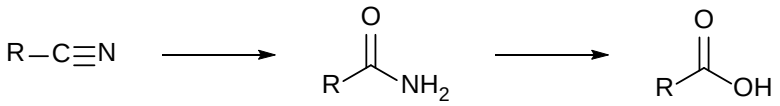
Scheme 2.9. *m*-Chloroperoxybenzoic acid oxidation of aminonitrile **173**. Reagents and conditions: a. *m*CPBA, DCM, 0 ^oC-RT, 2 h, 90%.

After successive acid and base washes to remove any residual *N*-butylimidazole and *m*-chlorobenzoic acid respectively, compound **174** was obtained as a yellow solid which was determined by HPLC analysis to be pure enough (>95%) to be used in the next step. However, high purity (>99% by HPLC) **174** was obtained after recrystallization from ethanol to give bright yellow crystals in 92% yield. No NMR spectroscopic data was obtained for compound **174** because of the paramagnetic broadening commonly observed for nitroxide containing compounds (as a result of

the presence of the free radical). However, FTIR, HRMS and HPLC analysis further supported the structural assignment and purity. The loss of the weak N-H stretch at 3326 cm^{-1} in the FTIR spectrum of **174** supported the transformation. In addition, a sharp C≡N stretch was observed at 2231 cm^{-1}. The mass of the molecular ion plus sodium $[M + Na]^+$ was observed at m/z 268 in the HRMS spectrum of **174**. The melting range determined for **174** was 200-201 ^oC.

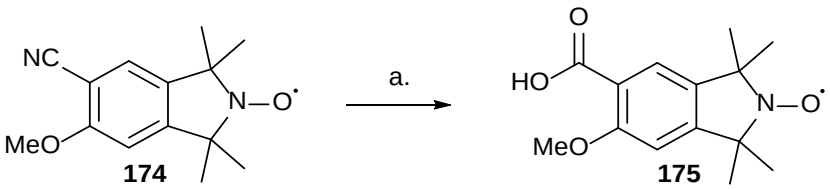
2.2.1.6. Synthesis of 5-Carboxy-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 163

Nitrile hydrolysis is a common synthetic method used to generate amides and carboxylic acids. In general, organic nitriles are hydrolyzed readily in strong acids (e.g. hydrochloric acid, sulfuric acid) or strong alkaline (sodium hydroxide) solutions. In either case, the reaction proceeds by first forming the amide which is further hydrolyzed, *in situ*, to the carboxylic acid (**Scheme 2.10**).



Scheme 2.10. Hydrolysis of nitriles to the corresponding carboxylic acids.

However, because nitroxides may disproportionate in strongly acidic solutions,[69] hydrolysing the nitrile nitroxide **174** by refluxing in acid was not attempted. Instead, nitrile nitroxide **174** was readily converted to the carboxylic acid derivative **175** by refluxing in 5 M sodium hydroxide solution (**Scheme 2.11**). Ethanol was used as a co-solvent in order to increase solubility of the nitrile **174**.



Scheme 2.11. Basic hydrolysis of nitrile nitroxide **174**. Reagents and conditions: a. 5 M NaOH, EtOH, reflux, 16 h, 89%.

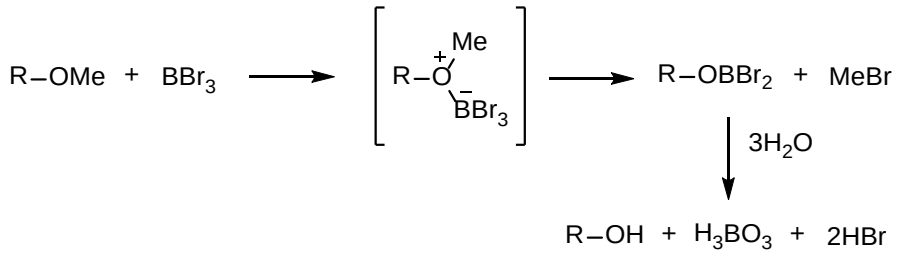
The desired carboxylic acid nitroxide **175** was obtained in 89% yield as a bright yellow crystalline solid following recrystallization from H_2O/EtOH. The FTIR spectrum of **175** confirmed the disappearance of the sharp C≡N stretch of the starting material **174** at 2231 cm^{-1}. The strong-broad O-H and strong C=O stretching frequencies, observed at 3400 and 1675 cm^{-1} respectively, supported the formation of a carboxylic acid functional group. The paramagnetic broadening effect of the nitroxide radical moiety of **175** limited the use of NMR spectroscopy for the characterization of compound **175**. The molecular ion plus sodium $[M + Na]^+$ and the molecular ion plus potassium $[M + K]^+$ for **175** were observed at m/z 287 and 303 respectively in the HRMS spectrum.

2.2.1.7. Synthesis of Salicylic Acid-TMIO 137 via Boron Tribromide Demethylation of 175

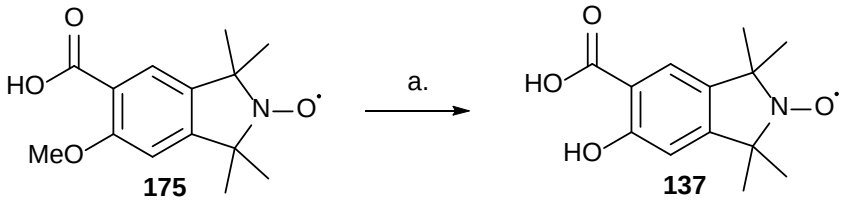
A variety of reagents are known to cleave ethers.[70] The most common ether cleavage reagents include the Bronsted acids (HCl, HBr), Lewis acids (aluminium and boron trihalides), basic reagents (alkali metals, hydroxides and amides), and miscellaneous reagents like the halogen trimethylsilanes. These reagents differ in their comparative reactivities for ether cleavage. They can have limitations with respect to the structure of the substrate and their tolerance to other functional groups present. Because of this, boron tribromide (BBr_3) is the reagent of choice for the cleavage of ethers. Its widespread use in this context is because the reactions are generally carried out under mild conditions (-78 ^oC to RT) and the cleavage usually does not affect other functional groups. Boron tribromide is a moisture-sensitive liquid that decomposes in air to give hydrobromic acid. As a strong Lewis acid, boron tribromide complexes to the ethereal oxygen atom and promotes the ether cleavage (**Scheme 2.12**).[71] For optimum ether cleavage, typically at least one molar equivalent of BBr_3 is used for each ether group and an extra equivalent is added for each functional group that contains lone pairs of electrons that could complex with BBr_3.

Thus, three molar equivalents of BBr_3 were added to a solution of carboxylic acid **175** in dichloromethane at -78 ^oC and the reaction mixture was allowed to return to room temperature while stirring overnight (**Scheme 2.13**). After work-up, which involved hydrolysis of the alkoxyborane with water and extraction with

dichloromethane, the desired salicylic acid-TMIO **137** was obtained as a yellow crystalline solid in 40% yield after recrystallization. The FTIR spectrum of the product displayed a medium-broad OH stretching frequency (3400-2500 cm^{-1}) along with a strong C=O stretch (1674 cm^{-1}). Compound **137** was of high purity (>99% pure by HPLC) with a melting range of 207-208 ^{o}C.

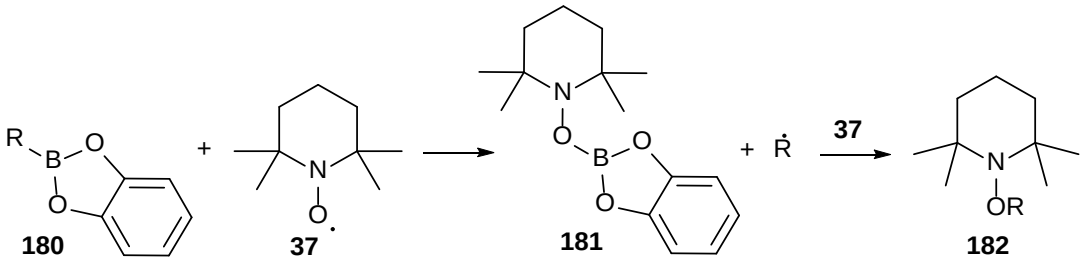


Scheme 2.12. Proposed mechanism for BBr_3-mediated ether cleavage.



Scheme 2.13. Boron tribromide demethylation of **175**. Reagents and conditions: a. BBr_3, DCM, -78 ^{o}C-RT, 18 h, 40%.

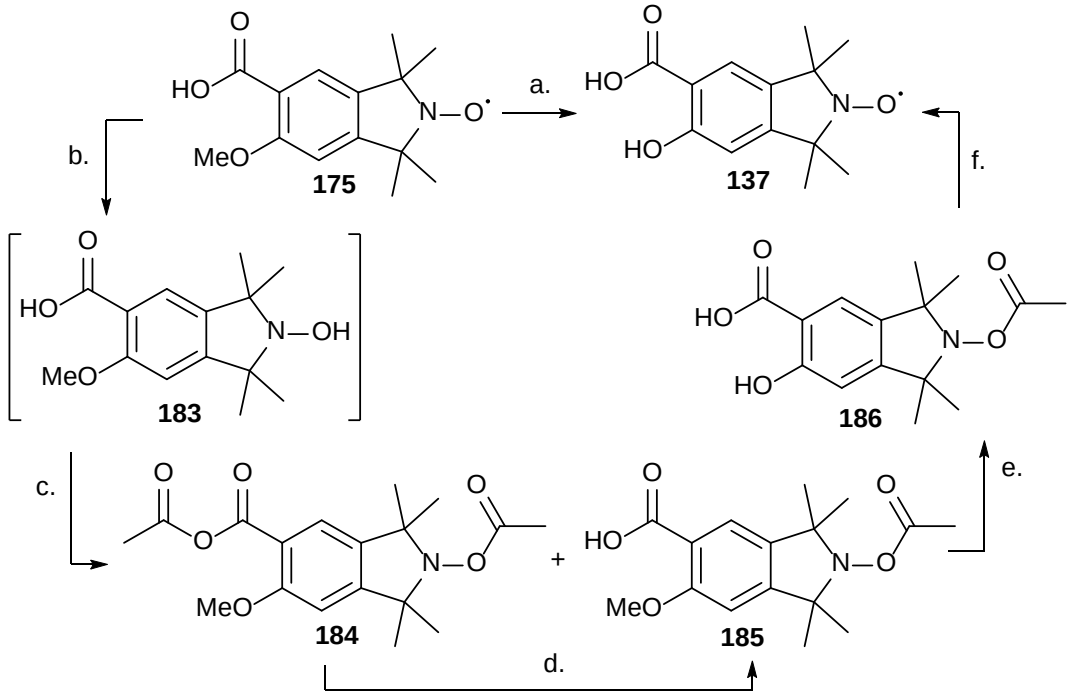
The low yield could be attributed to the potential formation of a complex between BBr_3 and the nitroxide moiety. Evidence to support the proposed nitroxide-BBr_3 complexation is reported by the work of Renaud *et al.* in which TEMPO was shown to complex with *β*-alkylcatecholboranes under mild radical hydroxylation conditions (**Scheme 2.14**).[72-75] Such nitroxide-BBr_3 complex formation could initiate multiple degradation pathways for both the starting material and the desired nitroxide compounds. To optimize the demethylation, an alternative approach was undertaken using a protecting group strategy for the nitroxide moiety.



Scheme 2.14. Reaction of *β*-alkylcatecholborane with nitroxide radicals. Adopted and modified from Renaud *et al*.[72,73]

2.2.1.8. Alternative Synthetic Route to Salicylic Acid-TMIO 137 from 175

One potential means to avoid such nitroxide-BBr_3 complexation (and, ultimately, to improve the yield) is to use a protecting group for the nitroxide moiety. The acetyl group is a simple nitroxide protecting group that is easily introduced and removed with no significant loss in yield.[51] The acetyl protection of **175** was carried out in a two-step one-pot synthesis (**Scheme 2.15**).



Scheme 2.15. Alternative route to **137** via protection of the nitroxide moiety of **175**. Reagents and conditions: a. 1 M BBr_3, DCM, -78 ^{o}C-RT, 1 d, 40%; b. H_2, Pd/C, THF, 15 min; c. TEA, AcCl, 0 ^{o}C-RT, 1.5 h, 96%; d. MeOH, 2 h; e. 1 M BBr_3, DCM, -78 ^{o}C-RT, 18 h, 85%; f. LiOH, H_2O/MeOH, 0 ^{o}C, RT overnight, PbO_2, 92%.

First, compound **175** was reduced to its corresponding hydroxylamine **183** via palladium-catalyzed hydrogenation. Then, the *in situ* generated hydroxylamine **183** was allowed to react with acetyl chloride in the presence triethylamine. The acetyl-protected intermediate **185** was obtained as a mixture with 10% of the anhydride **184** as determined by ^{1}H NMR spectroscopy. However, the anhydride **184** was easily hydrolyzed back to carboxylic acid **185** when the crude mixture was stirred in methanol for 2 hours. Compound **185** was obtained as a white crystalline solid in 96% yield after purification by chromatography and recrystallization. The singlets at 2.2 (3 protons), 4.1 (3 protons) and 10.8 ppm (br, 1 proton) observed in the ^{1}H NMR spectrum of **185** are characteristic protons of the acetyl, methoxy and carboxylic acid groups respectively. The two strong FTIR spectroscopy stretching frequencies at 1709 and 1772 cm^{-1} are consistent with the presence of the carboxylic acid and acetyl functionalities respectively.

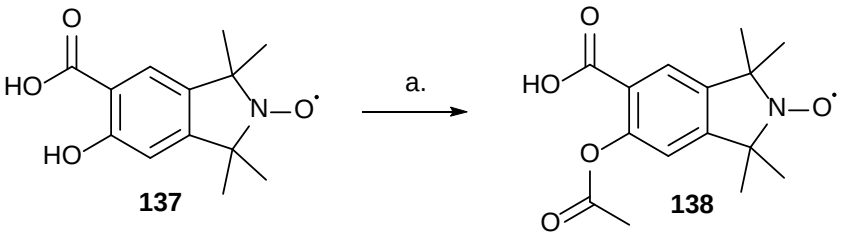
The acetyl-protected compound **185** was then subjected to the same mild boron tribromide mediated demethylation reaction conditions used in the preceding section (**Section 2.2.1.7**). This afforded the desired *N*-acetoxy salicylic acid **186** in excellent yield **(**85%). Evidence for successful demethylation of **185** was given by the disappearance of the methoxy proton and carbon peaks at 4.1 and 64 ppm in the ^{1}H and ^{13}C NMR spectra respectively. Though the phenolic proton could not be detected in the ^{1}H NMR spectrum (possibly due to strong hydrogen bonding with the *ortho*-carboxylic acid group or residual water) all characterization data obtained support the successful synthesis of **186**.

The acetyl-protecting group was removed following basic hydrolysis of compound **186** with lithium hydroxide to give the target salicylic acid-TMIO **137** in 92% yield. In comparison to the 40% yield obtained from direct demethylation of **175** (**Scheme 2.15.a**), the overall yield over the three steps (protection, demethylation and deprotection) was 75%.

Thus, the use of the acetyl protecting group clearly satisfies the prerequisites of a good protecting group: it was introduced easily in high yield, was stable under the boron tribromide demethylation conditions, did not interfere with the reaction, and was easily removed in high yield.

2.2.1.9. Synthesis of Aspirin-TMIO 138 from Salicylic acid-TMIO 137

The second target compound, aspirin-TMIO **138**, was readily synthesized by acetylating salicylic acid-TMIO **137** with acetyl chloride in the presence of triethylamine (TEA, **Scheme 2.16**). The target aspirin-TMIO **138** was obtained almost quantitatively as a bright yellow crystalline solid after recrystallization from water/ethanol. The acetyl and carboxylic acid C=O groups were observed as strong bands at 1765 and 1695 cm^{-1} respectively in the FTIR spectrum. The carboxylic acid stretching frequency was observed at 3400-2600 cm^{-1} as a medium broad band. Compound **138** was determined to be >95% pure by HPLC and decomposed at temperatures above 206-207 ^{o}C.



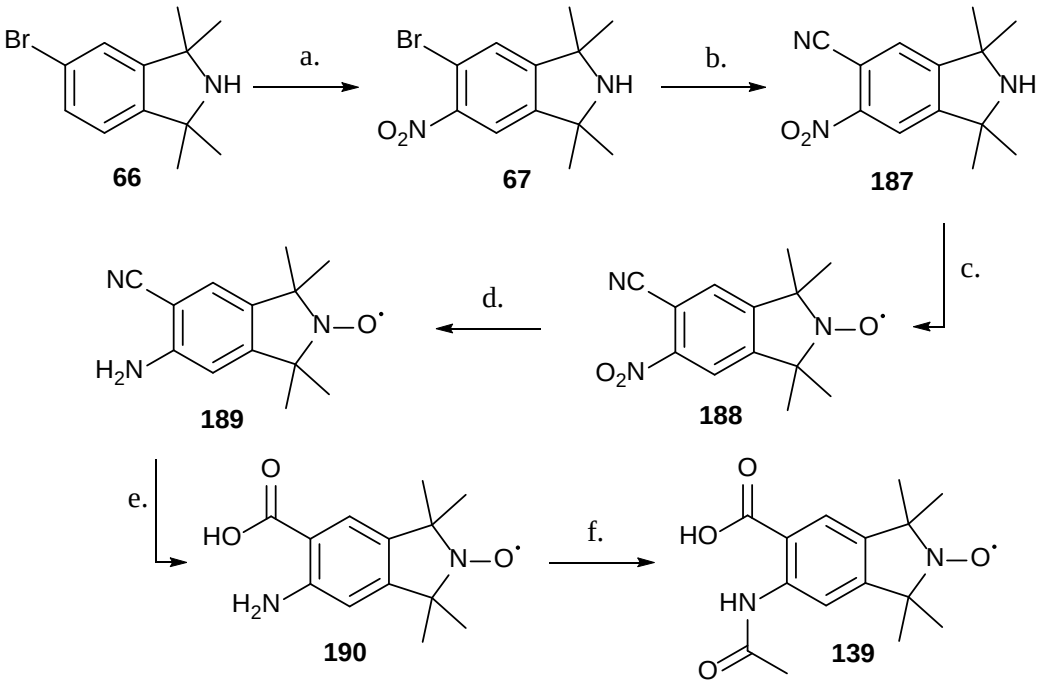
137 **138**

Scheme 2.16. Synthesis of aspirin TMIO **138**. Reagents and conditions: a. TEA, AcCl, THF, 0 ^{o}C-RT, 3 h, 91%.

With both salicylic acid and aspirin-containing TMIO compounds (**137** and **138**) in hand, the next section details the synthesis of the last target compound 6-acetylamino-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **139**.

2.2.2 Synthesis of 6-Acetamido-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 139

This section explores the synthesis of the final target compound in this chapter, *N*-acetylanthranilic acid-TMIO **139**. This analogue differs structurally from aspirin-TMIO **138** by having an *ortho*-*N*-acetyl group instead of an *ortho*-*O*-acetyl group. Such structural variation may provide means to compare the biological efficacy of the amide to the ester derivatives in inhibiting the MPO and COX enzymes. **Scheme 2.17** below outlines the synthetic route undertaken for the synthesis of compound **139**. Compound **190** was synthesized by following the literature procedure.[51]



Scheme 2.17. Synthesis of *N*-acetylanthranilic acid-TMIO **139**. Reagents and conditions: a. HNO_3, H_2SO_4, 0 ^oC, 5 h, 55%; b. $K_4[Fe(CN)_6]$, *n*Bulmi, CuI, toluene, 160 ^oC, 2 d, 68%; c. *m*CPBA, DCM, 0 ^oC-RT, 2 h, 93%; d. $SnCl_2.2H_2O$, HCl, 0 ^oC-RT, 3 h, 69%; e. KOH, H_2O/EtOH, reflux, 16 h, 75%; f. AcCl, TEA, THF, 0 ^oC, 3 h, 88%.

The starting bromoamine compound **66** was prepared in three steps from phthalic anhydride as detailed in **Section 2.2.1.1**. Nitration of aromatic compounds is generally a useful synthetic route to aromatic nitrogen containing compounds. It requires a strong electrophile, most commonly, a nitronium ion. The nitronium ion is formed by reacting concentrated sulfuric acid with nitric acid. When three equivalents of concentrated nitric acid were added to an ice-cold solution of bromoamine **66** in concentrated sulfuric acid, the reaction afforded the nitroamine **67** in 56% yield. Although excess nitric acid was used, only a single ring-nitration product was obtained as expected when a deactivating nitro group is added to the ring.

Using copper-catalyzed cyanation reaction conditions similar to those discussed in **Section 2.2.1.4**, a reaction mixture of compound **67**, potassium hexacyanoferrate (II), copper (I) iodide, and *N*-butylimidazole in toluene were refluxed for three days to

give crude cyano nitroamine **187**. Compound **187** was obtained as an off white solid in 68% yield after purification by flash column chromatography. The cyano nitroamine **187** was then oxidized to the nitroxide derivative **188** by allowing it to react with *m*CPBA in DCM. The nitroxide **188** was obtained as a bright orange solid in 93% yield after purification by column chromatography and recrystallization from ethanol. The melting range determined for **188** (230-231 ^{o}C) was in agreement with that reported in literature.[51]

The next step was to selectively reduce the nitro group of compound **188** to the amine derivative **189**. The reduction of nitro groups to their corresponding amine derivatives is most commonly carried out using tin chloride/hydrochloric acid ($SnCl_2$/HCl) or by catalytic hydrogenation. However, the nitrile group of **188** could also be reduced to the amine or the aldehyde derivative by hydrogenation. Although tin chloride reduces nitriles, such reduction reactions are usually carried out under anhydrous conditions with gaseous HCl.[76,77] Therefore in this case the nitro group of compound **188** was selectively reduced to the aminonitrile **189** using $SnCl_2$ in 2 M aqueous HCl solution. Although some nitroxides are reduced to amines by tin chloride, this did not occur with compound **188** under these aqueous conditions. The aminonitrile nitroxide **189** was obtained as a yellow solid in 69% yield after purification by flash column chromatography and recrystallization from ethanol.

Basic hydrolysis of the aminonitrile **189** with 5 M potassium hydroxide solution afforded the β-amino acid nitroxide **190** in 75% yield. The amino acid **190** was obtained as bright yellow crystals and decomposed at temperatures above 230 ^{o}C. This was consistent with that of the literature.[51]

The target *N*-acetylanthranilic acid-TMIO **139** was synthesized by acetylation of *β*-amino acid nitroxide **190** with acetyl chloride in the presence of triethylamine. The crude product was purified by recrystallization from water/methanol to give a yellow crystalline solid of *N*-acetylanthranilic acid-TMIO **139** in 88% yield. The presence of two strong carbonyl stretching frequencies at 1681 and 1590 cm^{-1} in the FTIR spectrum of **139** supported the presence of the carboxylic acid and acetamide groups respectively. In addition, a strong broad OH stretch was also observed (3340-2600 cm^{-1}) in the FTIR spectrum of **139**. Further structural confirmation for **139** was

obtained by the presence of the molecular ion plus proton $[M + H]^+$ mass at m/z 292 in the HRMS spectrum.

All of the synthesized compounds were determined to be of sufficient purity for biological screening, being >95% pure by HPLC.

2.2.3 Preliminary Biological Results

Following the synthesis of the target nitroxide-aspirin analogues, the next step in this project was to study the *in vitro* anti-inflammatory and antioxidant potentials of these new analogues. Preliminary antioxidant and anti-inflammatory activity studies on newly synthesized merged nitroxide-aspirin hybrids involved evaluating their inhibitory action on the MPO system and two isoforms of the COX enzyme (COX-1 and COX-2) respectively. Collaborators at the Australian Heart Research Institute (HRI) conducted both the initial antioxidant and anti-inflammatory experiments. Results discussed herein were obtained from studies conducted with the merged salicylic acid TMIO **137** and aspirin TMIO **138** hybrids. Compound **139** is currently being investigated for its antioxidant and anti-inflammatory effects.

2.2.3.1 Inhibitory Effects of Hybrid Salicylate-Nitroxides on the Myeloperoxidase Enzyme

The myeloperoxidase (MPO)-mediated hypochlorous acid production system is well-known therapeutic target due to its involvement in the progression of neurodegenerative and chronic inflammatory diseases.[1,4] Nitroxides and other potent antioxidants have been previously shown to inhibit the MPO system.[78-82] In these studies, the MPO system used to produce the strong hypochlorous acid oxidant comprised of MPO (10–500 nM), Cl^- (100 mM) and H_2O_2 (10–50 μM).[83] The enzyme was incubated either alone (control) or with each nitroxide and the reactions were initiated by the addition of H_2O_2. The reactions were performed at 25 ^oC at or near the physiological pH of 7.4. Each experiment was performed in the presence of taurine and a bovine liver catalase was used to stop the reactions. Taurine is a β-amino acid which rapidly traps hypochlorous acid produced by the MPO system to form the stable oxidant taurine chloroamine (Tau-Cl). Taurine chloroamine subsequently reacts with 5-thio-2-nitrobenzoic acid (which has a strong absorbance

at 412 nm) to produce the colourless compound 5,5'-dithio-bis(2-nitrobenzoic acid) **199** (DTNB). The MPO inhibition activity of each compound is inversely proportional to the decrease in DNB absorbance which in turn is proportional to the amount of Tau-Cl generated. Aspirin and salicylic acid-TMIO based compounds **137** and **138** were evaluated along with the parent aspirin compound **136**.

As shown in **Figure 2.5** below, the parent aspirin **136** displayed no inhibitory effect on the MPO system even at higher concentration. With respect to the hybrid nitroxides, neither CTMIO **70**, salicylic acid-TMIO **137**, nor aspirin-TMIO **138** could inhibit the production of hypochlorous acid at 10 μM concentrations (**Figure 2.6**, **Figure 2.7** and **Figure 2.8**). At concentrations ≥ 100 μM however, a gradual decrease in the amount of Tau-Cl generated was observed for each hybrid nitroxide.

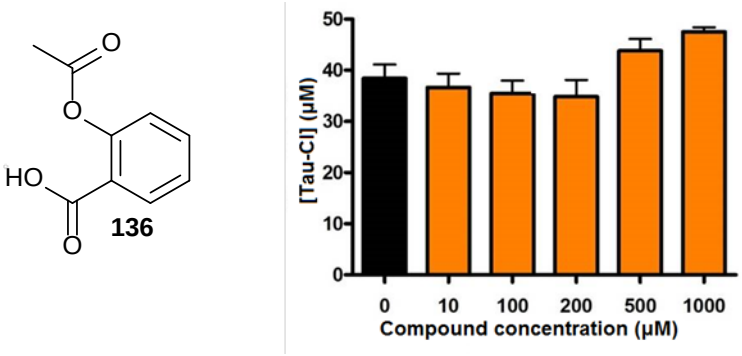


Figure 2.5. Parent aspirin 136 inhibitory action of MPO-system.

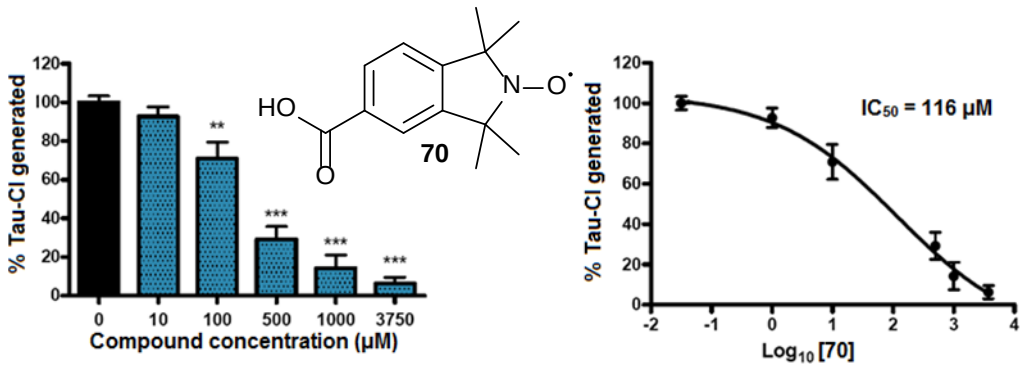


Figure 2.6. CTMIO **70** inhibition of MPO-system (*** denotes marked inhibitory effect).

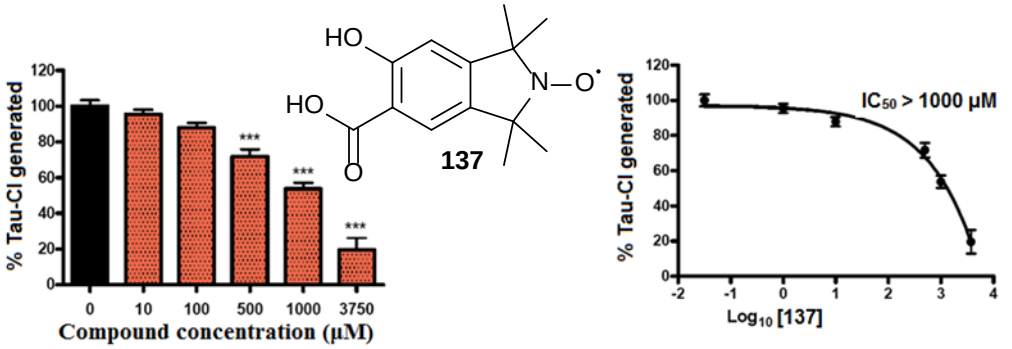


Figure 2.7. Salicylic acid-TMIO **137** inhibition of MPO (*** denotes marked inhibitory effect).

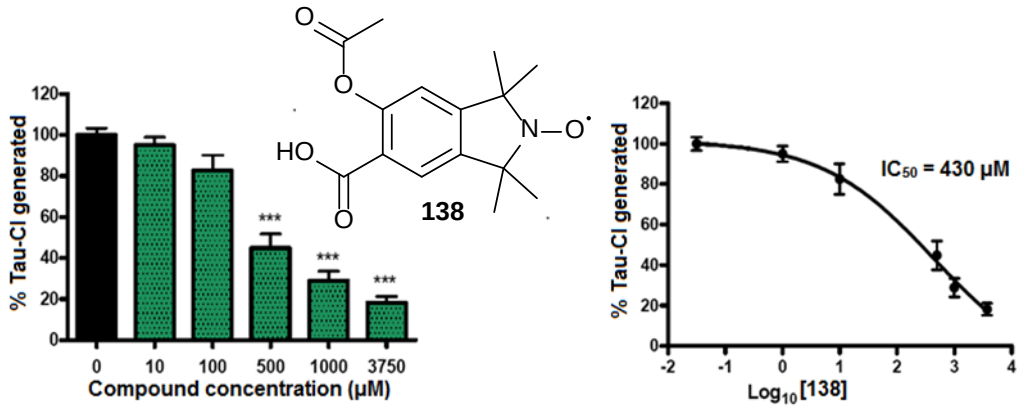


Figure 2.8. Aspirin TMIO **138** inhibition of MPO (*** denotes marked inhibitory effect).

The IC_{50} values from each dose-response curve were 116, 430 and >1000 μM for CTMIO **70**, aspirin-TMIO **138** and salicylic acid-TMIO **137** respectively. The results indicated a dose-dependent inhibitory effect of the hybrid nitroxide salicylate compounds on the MPO system.

The fact that these novel nitroxides do inhibit the myeloperoxidase system albeit at higher concentration was encouraging and demonstrated a clear antioxidant capacity for these hybrids. The inhibitory effect however is modest when compared to amino-TEMPO nitroxide (currently the most potent nitroxide inhibitor of MPO reported) which has an IC_{50} value of 1 μM.[78] This is likely to be size-related, since previous studies have shown that the extent of nitroxide-mediated MPO inhibition largely depends on the structure of the nitroxide (which governs access to the enzyme active

site).[78] Nevertheless, these results do support, at least in part, our hypothesis that these novel analogues may have potential to function as therapeutic antioxidant in biological environments with particular targets such as the gastrointestinal mucosa linings.

The next section details some preliminary COX inhibition results for the novel hybrid compounds.

2.2.3.2 Inhibitory Effects of Hybrid Salicylate-Nitroxides on Cyclooxygenase Enzymes

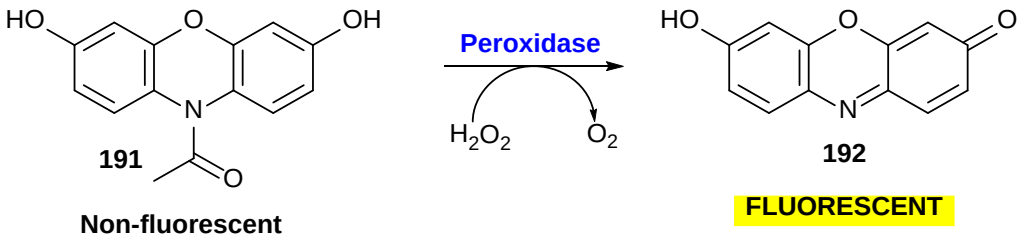
In order to evaluate the anti-inflammatory action of the novel nitroxide analogues, inhibitory studies were carried out on both isoforms of the COX enzyme, COX-1 and COX-2.

2.2.3.2.1. COX Inhibitory Studies using the Amplex Red Fluorescent Inhibitor Screening Assay

Preliminary studies were carried out using the COX Fluorescent Inhibitor Screening Assay Kit (Cayman Chemical, Item No. 700100). Following a phospholipase H_2-catalyzed liberation of arachidonic acid from the phospholipids of the cell membrane, each isoform of the COX enzyme can cyclize arachidonic acid and add the 15-hydroperoxy group to form prostaglandin G_2 which in turn decomposes to various prostanoids involved in both normal cell function and inflammation (**Figure 2.2**). The peroxidase component of the COX enzyme uses a range of substrates to provide the required pair of electrons.[84] The fluorescent inhibitor screening assay is designed to monitor the reactions that occur at the peroxidase active site of the COX enzyme. It uses 10-acetyl-3,7-dihydroxyphenoxazine **191** (ADHP or Amplex Red) as a fluorogenic peroxidase substrate (**Scheme 2.18**).[85] In the presence of peroxidase, ADHP (a nonfluorescent compound) is converted to the highly fluorescent resorufin that has maximum excitation and emission of approximately 571 and 585 nm respectively.

The COX inhibition experiments were carried out in duplicate wells, in duplicate experiments (with the exception of TEMPO, n=1). As expected, aspirin **136**

significantly inhibited the COX-1 enzyme at 750 μM (**Figure 2.9**). TEMPO **38**, at 750 μM, displayed some COX-1 inhibition but not as much as aspirin. TEMPO **38** in combination with aspirin **136** showed an even higher inhibitory action of the COX-1 enzyme.



Scheme 2.18. Peroxidase/H_2O_2 oxidation of Amplex Red. Peroxidase converts ADHP **191** to resorufin **192**, a highly fluorescent compound which can be analyzed with Ex/Em 571/585 nm.

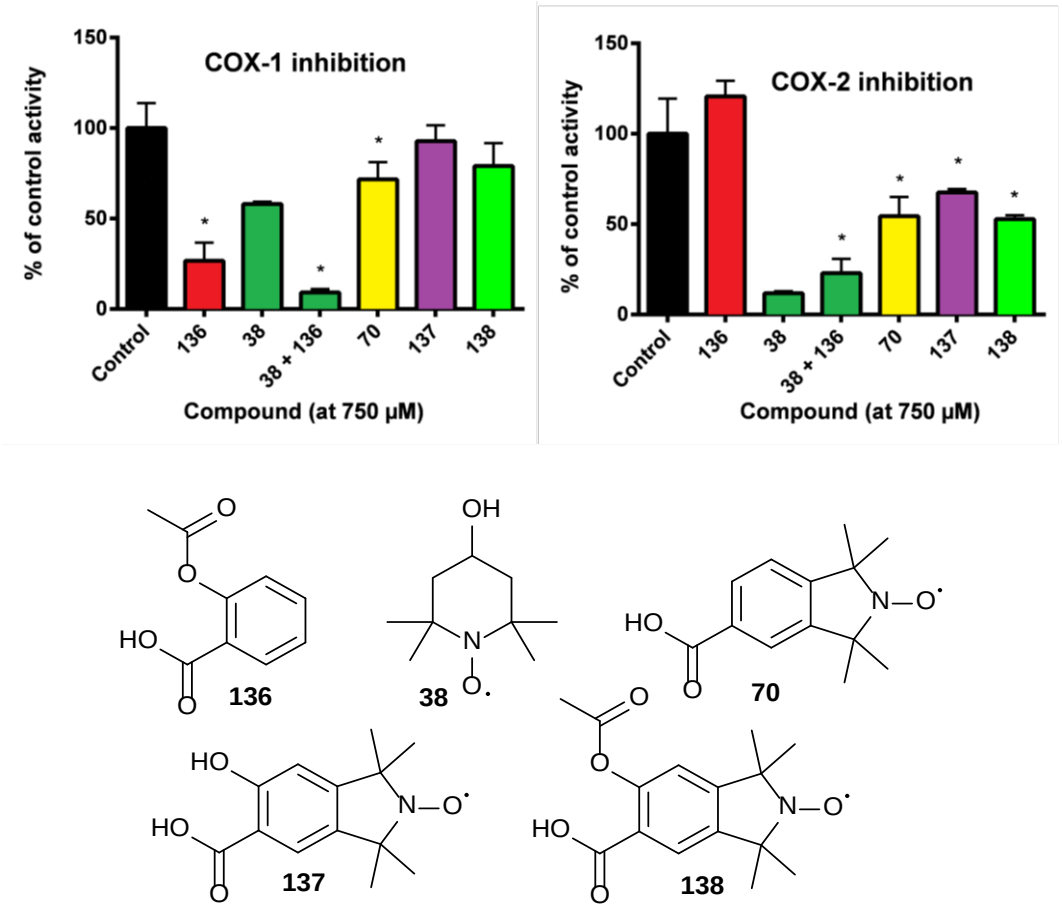


Figure 2.9. COX inhibitory studies of nitroxides using the Amplex Red assay (* denotes marked inhibitory effect).

Although CTMIO **70** displayed moderate inhibitory action of the COX-1 enzyme, neither the salicylic acid-TMIO **137** nor the aspirin-TMIO **138** hybrids had any significant inhibitory effect on the COX-1 enzyme. From the COX-2 study, TEMPO **38** had the most significant inhibitory effect at 750 μM. However, CTMIO **70** and the hybrid salicylic acid and aspirin nitroxide compounds (**137** and **138**) also displayed good COX-2 inhibition, but not as much as TEMPO **38**.

Although the results seem promising, the important caveat in this study is that aspirin (known to inhibit both COX isoforms) did not inhibit the COX-2 enzyme in this specific assay.[86] Also, the combination of aspirin **136** with TEMPO **38** appears to have a lesser inhibitory effect than TEMPO **38** alone. Furthermore, the significant inhibitory effect of TEMPO **38** on both COX isoforms was surprising as TEMPO **38** has no structural functionality, besides the nitroxide moiety, that would give rise to this inhibitory capacity.

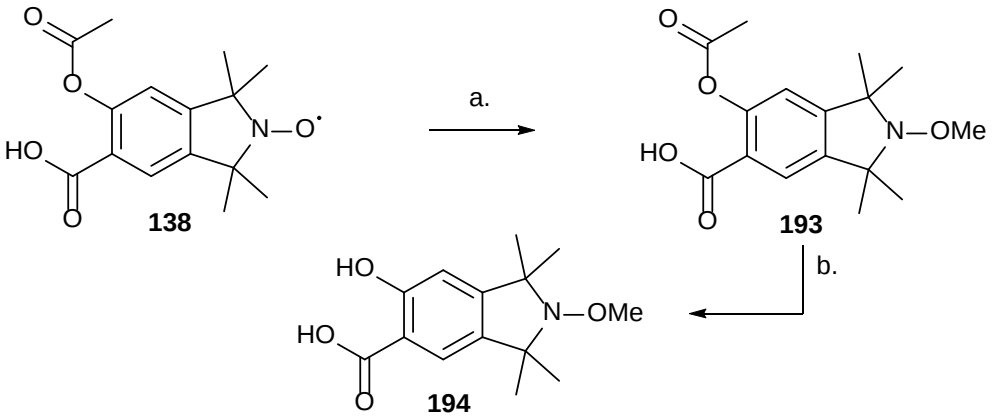
One possible explanation for this observation could be that the nitroxide radical centre is interfering with the Amplex Red assay and thus giving a false positive COX inhibitory effect. In a recent study, Serrano *et al.* observed that dietary antioxidants (such as gallic acid, epicatechin, quercetin, vanillic acid and ascorbic acid) could interfere with the Amplex Red probe/ horseradish peroxidase assay.[87] Although the mechanism for such interactions is complex, they proposed that it involves direct redox interactions of dietary antioxidants with the ferric centre of the peroxidase enzyme. This subsequently results in the production of antioxidant radicals which in turn act as competitive substrates for the peroxidase enzyme.

Similarly, the antioxidant nitroxide moiety could be acting as a substrate that competes with the non-fluorescent ADHP **191** for the peroxidase enzyme of the Amplex Red assay. This leads to less resofurin production and therefore less fluorescence would be observed, thus explaining the strong inhibitory effect of TEMPO **38** that was observed. It is likely in such a case that the nitroxide moiety is either reduced to the hydroxylamine or oxidized to the oxoammonium derivative. The case of nitroxide reduction (or oxidation) can depend on the nitroxide ring class. This is a possible explanation for the observed difference between COX inhibitory effect of TEMPO **38** (a piperidine type nitroxide) and the isoindoline hybrids

(CTMIO **70**, salicylic acid TMIO **137** and aspirin-TMIO **138**). To determine the potential for the nitroxide to interfere with the Amplex Red assay, the non-radical methoxyamines were synthesized and assessed.

2.2.3.2.2. Synthesis of Alkoxyamine Derivatives of Salicylic Acid TMIO and Aspirin TMIO

To assess the possibility that the nitroxide moiety interferes with the Amplex Red reaction, additional experiments were carried out, this time with the nitroxide moiety of each analogue converted to the corresponding methoxyamine derivative. These derivatives were synthesized as outlined in **Scheme 2.19** below. Methyl radicals generated by Fenton chemistry (from dimethyl sulfoxide, ferrous ions and hydrogen peroxide) were allowed to react with aspirin TMIO **138** to afford the diamagnetic *N*-methoxyamine derivative **193**.



Scheme 2.19. Synthesis of methoxyamine derivatives of salicylic acid-TMIO and aspirin-TMIO. Reagents and conditions: a. H_2O_2, $FeSO_4.7H_2O$, DMSO, 1 h, 64%; b. 2 M NaOH, 3 h, 96%.

In the ^{1}H NMR spectrum of **193**, the singlets at 2.2 ppm and 3.8 ppm, each integrating for 3 protons, represent the OAc and N-OMe protons respectively. The carboxylic acid proton of **193** resonated as a weak, broad singlet at 10.7 ppm. In the FTIR spectrum of **193**, two strong carbonyl (C=O) stretching frequencies were observed at 1690 and 1689 cm^{-1} which account for the acetyl and carboxylic acid groups respectively. A strong, broad O-H band stretching from 3400 to 2800 cm^{-1} of the COOH group was also observed.

Basic hydrolysis of **193** afforded the salicylic acid derivative **194** quantitatively. Although the phenolic proton could not be assigned in the ^{1}H NMR spectrum (possibly due to a strong hydrogen bonding with the *ortho* carboxylic acid group or residual water), other characteristic data obtained to support the successful synthesis of **194** included FTIR and ^{13}C NMR spectra and HRMS.

Initial attempts to synthesize the alkoxyamine **194** directly from the nitroxide counterpart **137** (salicylic acid-TMIO) using the Fenton chemistry produced a mixture of the desired product (≤ 40 yield, after purification) and other uncharacterized side products. This could be attributed to a likely reaction of the phenolic groups of **137** and **194** with the methyl radicals.

The methoxyamine derivatives **193** and **194** were tested for their inhibitory activity of the COX enzyme along with their nitroxide counterparts using the Amplex Red peroxidase fluorescence assay kit. As shown in **Figure 2.10** below, at 700 μM concentrations, neither alkoxyamine derivatives **193** or **194** had any significant inhibitory effect on both COX-1 and COX-2 enzymes.

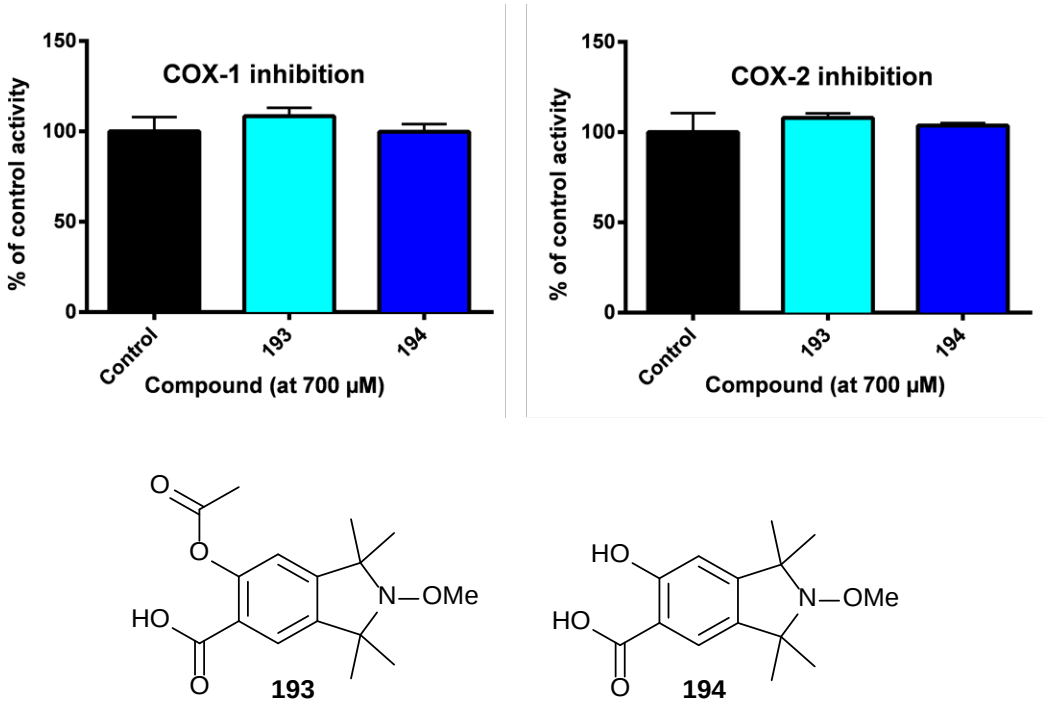


Figure 2.10. COX inhibition results for methoxyamine derivatives **193** and **194**.

While this result reinforced the claim for a potential nitroxide interference with the Amplex Red assay, it is difficult to determine whether any interference predominantly gave rise to the observed COX inhibitory activity by TEMPOL and the hybrid salicylates and that the mechanism of inhibition of nitroxides (TEMPO **38**, CTMIO **70**, salicylic acid TMIO **137** and aspirin TMIO **138**) arises at least in part, perhaps in a large part, from their antioxidant activity. Thus, the measured inhibitory effect could either arise from the nitroxide interference with the Amplex Red assay or from actual inhibitory effect from the nitroxide moiety, or a combination of both. In other words, the Amplex Red fluorescence assay could be measuring the COX inhibitory activity of hybrid nitroxide compounds while at the same time its peroxidase component is involved in a radical-mediated interaction with the nitroxide moiety.

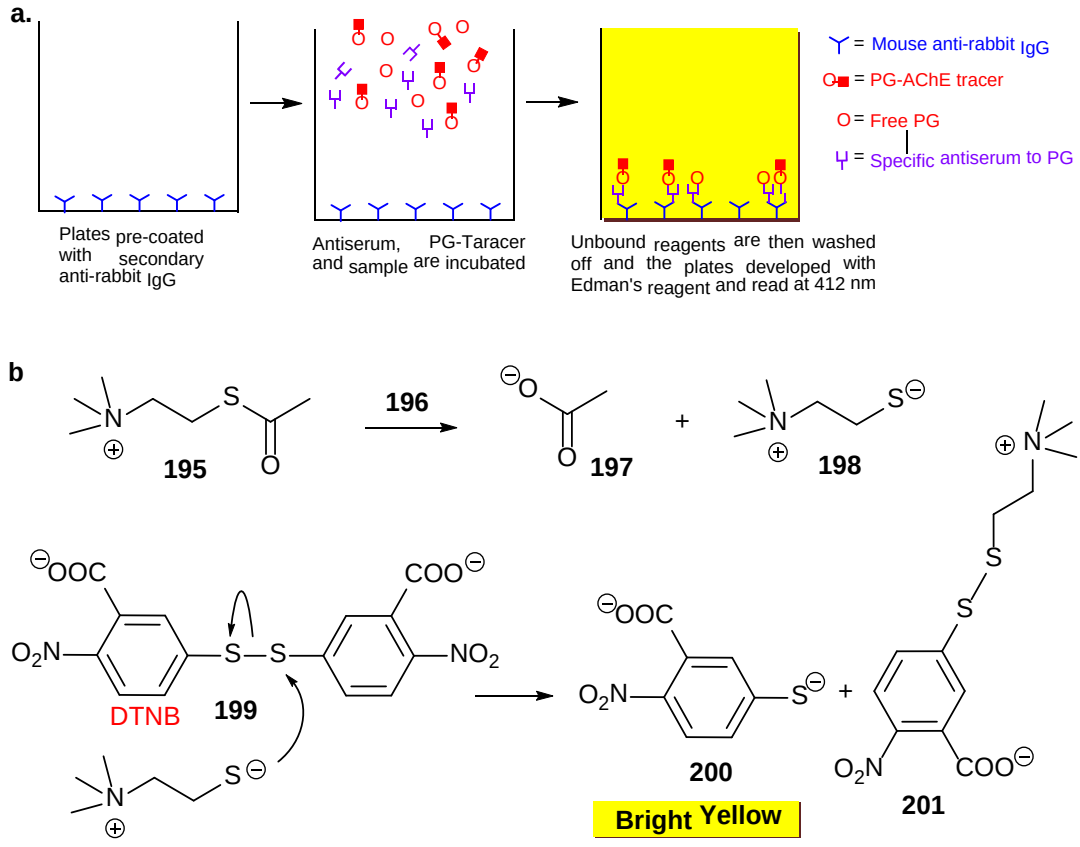
Although the Amplex Red results are not conclusively showing NSAID character, they still however, confirm the potent redox character of these compounds. Although the results link the significant COX inhibition observed for TEMPO (and other nitroxides) to the redox interference of the nitroxide moiety, the extent of such interference remains unknown. Thus, using other COX inhibitory assays with no prior reported antioxidant interference is required. One assay suitable for this purpose is the COX (ovine) inhibitor screening assay kit in which the enzyme-linked immunosorbent assay (ELISA) is employed to quantify the COX-induced PGs production. The next section discusses the technique and results obtained with the ELISA method.

2.2.3.2.3. COX Inhibition Studies using the Enzyme-Linked Immunosorbent Assay (ELISA)

Other methods used to quantify the inhibitory action of potent anti-inflammatories on the COX enzyme include monitoring the rate of oxygen consumption (from the double oxygenation of the arachidonic acid) or by quantitative measurement of the prostaglandins produced during the COX reaction. The most convenient COX (ovine) inhibitor screening assay however uses ELISA to directly measure the quantity of each PG produced from the COX reaction.

This high throughput method uses tin (II) chloride to reduce the COX-derived PGH_2 non-enzymatically to a variety of primary prostanoids. Before the COX reaction is started (by the addition of arachidonic acid), target compounds are pre-incubated with both isoforms of the COX enzyme. The prostanoid products derived from the COX reaction are then quantified using ELISA. The ELISA kit uses a specific antiserum that binds to all major bioactive PG products (**Scheme 2.20**).[88] Also, it requires a constant concentration of PG-acetylcholinesterase **196** (AChE) tracer (obtained by covalent coupling of PG to pure AChE) that competes with PG (produced from COX reaction) for a limited amount of PG antiserum.

Absorbance α [PG-AChE tracer] α 1/[PG products from COX reaction]



Scheme 2.20. ELISA method for detecting COX activity by quantification of PGs: a. Schematic of the quantification process; b. AChE (**196**)-catalyzed hydrolysis of acetylthiocholine **195** and subsequent reduction of Ellman's reagent.

The PG-AChE tracer which is bound to the antiserum is quantified by reaction with Ellman's reagent. The Ellman's reagent contains acetylthiocholine **195** and 5,5'-

dithio-bis(2-nitrobenzoic acid) **199** (DTNB) substrates. Acetylthiocholine **195** is hydrolyzed by AChE **196** to produce thiocholine **198** which in turn reacts with DTNB **199** to produce 5-thio-2-nitro-benzoic acid **200**. 5-Thio-2-nitro-benzoic acid **200** has a strong absorbance at 412 nm. The intensity of the coloured **200** is proportional to the amount of the tracer bound to the antiserum which is inversely proportional to the amount of PG produced from the COX reaction.

Results obtained from duplicated experiments with pre-incubation times of 2 minutes are shown in **Figure 2.11** below. At 10 μM, neither the parent aspirin nor the novel nitroxide derivatives could inhibit the COX-1 enzyme. At 100 μM and 500 μM, aspirin **136** showed strong inhibitory effects on COX-1 as expected.

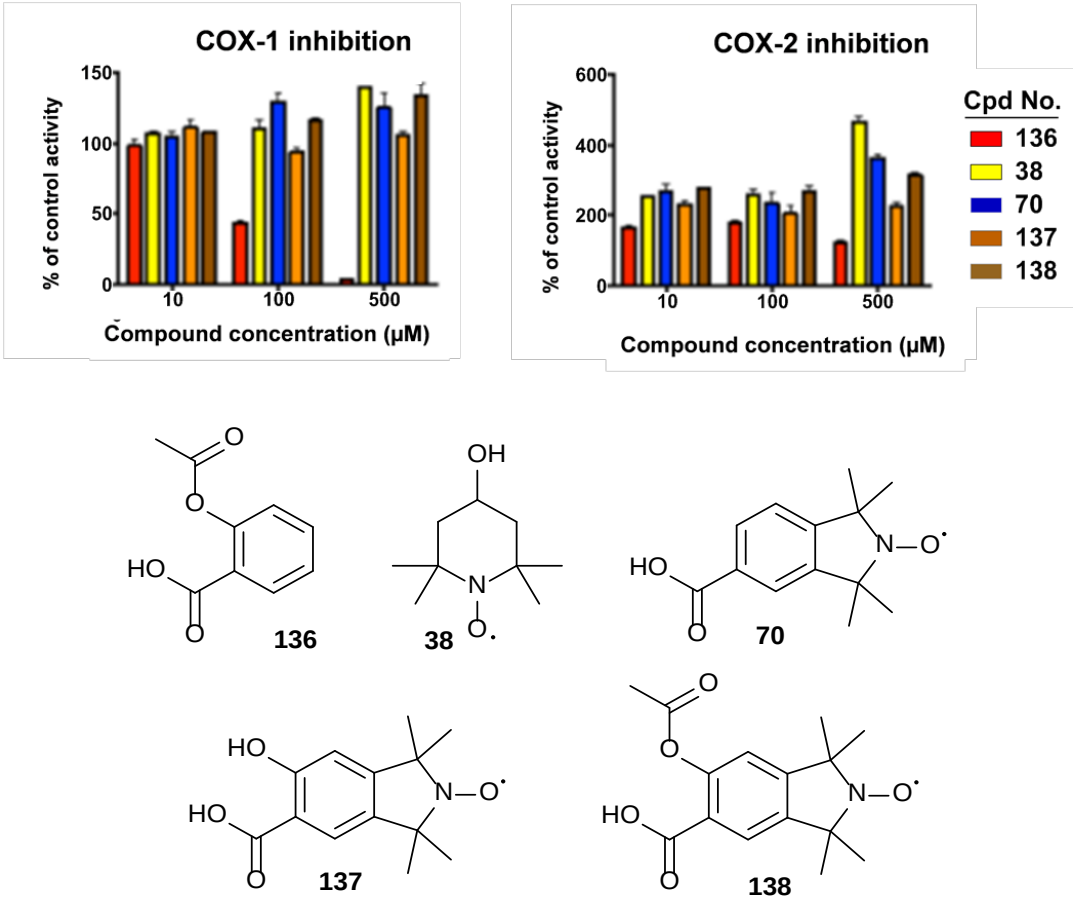


Figure 2.11. COX inhibitory action of nitroxides after 2 minutes incubation (Cpd No. refers to compound numbers).

However, none of the nitroxide compounds (**38**, **70**, **137** and **138**) displayed any significant inhibitory effects towards either COX isoform even at 500 μM. Most

notably, TEMPO **38**, which with the Amplex Red assay indicated strong inhibitory effects of both COX isoforms, had no effect on both COX enzymes with this assay. This further supports the proposed redox interaction of the nitroxide compounds with the Amplex Red assay.

Several studies have now determined that aspirin irreversibly deactivates the COX enzyme by rapidly blocking the entry of arachidonic acid to the COX active site by acetylating amino acid serine 530.[44,89] Unlike aspirin however, most NSAIDs such as indomethacin and celecoxib harness their inhibitory action by either hydrogen bonding, ionic, van der Waals or hydrophobic interactions (or a combination) with distinct amino acid residues within the long, narrow hydrophobic region of the COX enzyme.[45] Thus, most NSAIDs are slow time-dependent inhibitors of the COX enzyme.

To test whether the hybrid nitroxide derivatives could be slow time-dependent COX-inhibitors, further COX inhibitory experiments were therefore conducted with longer pre-incubation times (20 min, **Figure 2.12**). As expected, aspirin **136** inhibited both COX isoforms at 20 minutes. Neither aspirin-TMIO **138** nor the methoxyamine derivative **193** showed any inhibitory activity on the COX enzymes. Likely, the constrained nature of the hydrophobic inhibition region of the COX enzyme limits access to serine 530 by the acetyl group of aspirin TMIO **138** through steric constraints.[89] In contrast, salicylic acid-TMIO **137** possessed moderate inhibitory action on both COX enzymes after a 20-minute incubation. Such moderate inhibitory action after longer incubation is promising and suggests that the hybrid nitroxide compounds could be slow-time dependent COX inhibitors.

Hence, prolonging the incubation (up to hours) may be necessary to fully deduce the anti-inflammatory action of these hybrid nitroxides. Notably, the methoxyamine derivative of salicylic acid-TMIO **194** had very little inhibitory effect on the COX enzymes. Thus the moderate COX inhibitory action displayed by salicylic acid-TMIO **137** could arise from the antioxidant nitroxide moiety instead of the salicylate group. Although the methoxyamine derivatives (**193** and **194**) give a rough estimate of the COX inhibitory effect in the absence of the nitroxide moiety, it is important to

note that they are slightly different compounds and may have different binding and solubility characteristics.

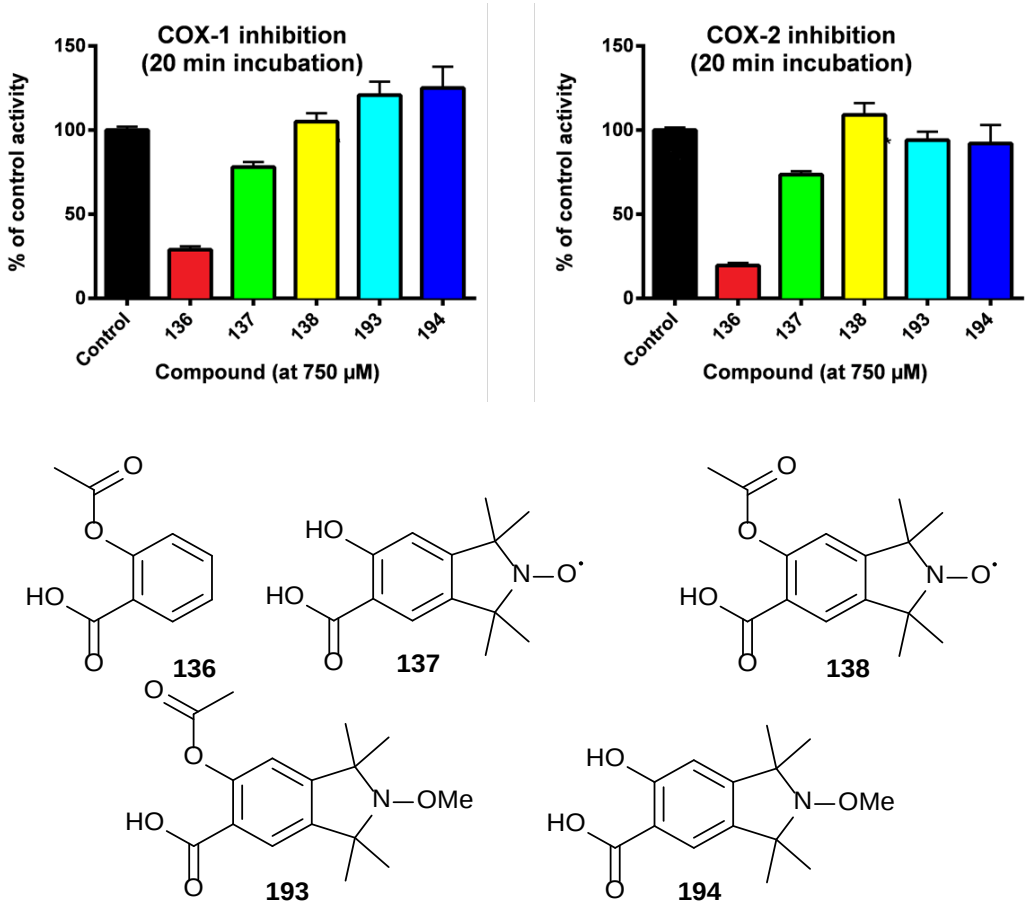


Figure 2.12. COX Inhibitory action of nitroxides (20 min incubation).

Thus an alternative explanation is that the conversion of the more polar nitroxide to the less polar methoxyamine interferes with a critical element required for binding in the COX active site. The restricted nature and critically important electron density of the hydrophobic region of the COX active site means the alkoxyamine derivatives could have a different mode of interaction in the inhibitory region in comparison with parent nitroxides. Further studies using non-radical secondary amines may help to elucidate this point.

2.3 Summary of Results

Using the pharmacophore hybridization strategy, three novel, potential dual action nitroxide-salicylate hybrids were successfully synthesized by overlapping similar structural subunits of the parent molecules. The following preliminary conclusions can be deduced from the results obtained from all biological evaluations (on both MPO and COX) performed to date.

CTMIO **70**, salicylic acid-TMIO **137** and aspirin-TMIO **138** showed moderate inhibition of MPO activity. This reinforces one of their several modes of antioxidant capacity (that may include their SOD mimetic action, lipid peroxidation inhibition, and radical scavenging potential), despite their moderate IC_{50} values when compared to amino-TEMPO **76**. This is likely to be related to the nitroxide structure (size and ring class).

Studies to evaluate the anti-inflammatory actions of the hybrid nitroxide-salicylate derivatives were first carried out with the PG-Amplex Red COX inhibitor assay. Initial results obtained from these experiments suggested potent COX inhibitory effect of the hybrid nitroxide-salicylate derivatives and, in particular, TEMPO **38**. However, further studies with the alkoxyamine derivatives (**193** and **194**) of these hybrids indicated that the nitroxide moiety is likely acting as a substrate for the PG-Amplex Red assay, thus leading to a false positive result. Since dietary antioxidants are known to interfere with the PG-Amplex Red assay, the proposed similar nitroxide reaction with the PG-Amplex Red assay supports the antioxidant action of the novel nitroxide compounds detected by this methodology. To obtain more insight into the COX inhibitory effect of these compounds, further experiments were conducted with the ELISA assay. The results indicate that salicylic acid-TMIO **137**, but not aspirin-TMIO **138**, had moderate inhibitory effect on both isoforms of the COX enzyme but only after prolonged incubation (20 min). This, therefore, suggests that the hybrid nitroxide-salicylate derivatives are likely to be slow-time dependent COX inhibitors.

The preliminary results for the inhibition of both MPO and COX enzymes by nitroxide compounds are encouraging. They indicate that combining antioxidant nitroxides with known pharmacophores (such as aspirin) is, evidently, an attractive

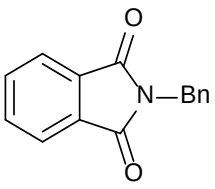
approach for developing potent novel dual action (antioxidant and anti-inflammatories) therapeutics for chronic inflammatory diseases. However, further studies are still required in order to have a clear understanding of the structure-activity relationships for these hybrid nitroxide agents. The next chapter, through pharmacophore hybridization, further expands on the dual drug design approach by exploring the synthesis and biological evaluation of a different series of nitroxide-NSAID conjugates including compounds **140-146**.

2.4 Experimental

The syntheses reported in this project were carried out at the Queensland University of Technology (QUT), Brisbane, Australia.

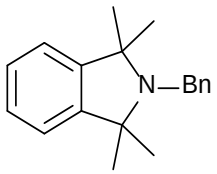
All air-sensitive reactions were carried out under ultra-high pure argon. Diethyl ether and toluene were dried by storing with sodium wire. All other solvents were dried using the Pure Solv Micro 4 L solvent purification system (PSM-13-672). Solvents used for extractions and silica gel column chromatography were AR grade. Crystalline $K_4[Fe(CN)_6]\cdot 3H_2O$ was ground to a fine powder and then dried at 80 °C at 0.5 Torr for 10 h. All other reagents were purchased from commercial suppliers and used without further purification ^1H and ^{13}C NMR spectra were recorded with Bruker Avance 600 MHz, 400 MHz or Varian 400 MHz spectrometers and referenced to the relevant solvent peak. HPLC was performed with a HP Agilent 1100 HPLC instrument. HRMS was performed with an Agilent accurate mass QTOF LC-MS spectrometer. Formulations were calculated by elemental analysis using a Mass Lynx 4.0 or Micromass Opus 3.6 instrument. FTIR spectra were recorded with a Nicolet 870 Nexus Fourier Transform Infrared Spectrometer equipped with a DTGS TEC detector and an ATR objective. Melting points were measured by Buchi Melting Point M-565 apparatus.

2.4.1 Synthesis of N-benzylphthalimide 168



Phthalic anhydride **167** (10 g, 67.51 mmol, 1 equiv.) was dissolved in glacial acetic acid (50 mL). To this was added benzylamine (10.94 mL, 101.3 mmol, 1.5 equiv.) and the resulting solution refluxed for 1 hr. The reaction mixture was cooled to room temperature, poured onto ice/water (150 mL) and stirred to give a white precipitate which was then filtered and recrystallized from ethanol to give fluffy white crystals of *N*-benzylphthalimide **168** (15.7 g, 98%). Mp. 116 ^{o}C (Lit.,[50] 115-116 ^{o}C). ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 4.87 (s, 2 H, CH_2), 7.27-7.36 (m, 3 H, Ar-H), 7.46 (d, 2 H, *J* = 7.2 Hz, Ar-*H*), 7.73 (d, 2 H, *J* = 5.6 Hz, 4 Hz, Ar-*H*), 7.87 (d, 2 H, *J* = 5.6 Hz, 4 Hz, Ar-*H*) . ^{13}C NMR (100 MHz, $CDCl_3$) δ = 41.62 (*C*H_2), 123.35 (Ar-*C*), 127.34 (Ar-*C*), 128.63 (Ar-*C*), 128.69 (Ar-*C*), 132.14 (Ar-*C*), 133.99 (Ar-*C*), 136.39 (Ar-*C*), 168.05 (*C*=O). The obtained NMR data were consistent with that previously reported in the literature.[50]

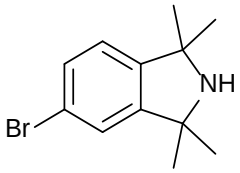
2.4.2 Synthesis of N-Benzyl-1,1,3,3-tetramethylisoindoline 169



Magnesium turnings (37.5 g, 1.48 mol, 7 equiv.) and all glassware were placed in an oven at 80 ^{o}C and dried overnight before the reaction. The Mg turnings with a couple of iodine crystals were placed in a 2 L three-neck flask fitted with a thermometer, a dropping funnel, and a Dean Stark apparatus connected, through a condenser, to an Ar and vacuum lines. The flask was heated carefully with a heat gun under high vacuum to evacuate remaining moisture and then allowing the flask to cool to RT. The flask was placed under Ar and dry Et_2O (500 mL) was added through a dropping funnel. The reaction mixture was stirred gently and few drops of the MeI (78.7 mL, 1.26 mol, 6 equiv.) were added to start the reaction. As the reaction mixture began to turn grey and the Et_2O started to boil, the remaining MeI was added slowly at a steady rate to keep the Et_2O boiling constant. After addition of all the MeI, complete formation of the MeMgI Grignard reagent was indicated by the time when the Et_2O stopped boiling. A solution of *N*-benzylphthalimide **168** (50 g, 211 mmol) in dry toluene (500 mL) was added with care and the resulting solution was heated to 80 ^{o}C to remove the Et_2O. The solution was refluxed for 4 h and then allowed to cool to

RT. Hexane (400 mL) was added and the mixture filtered through Celite with the solid residue washed several times with hexane and filtered. Air was bubbled through the filtrate until it was almost dry. The filtrate was dissolved in hexane and filtered through a basic alumina column to separate the product from the purple intractable material. The column was washed several times with hexane and the combined filtrate was concentrated *in vacuo*. The residue was recrystallized from methanol to give white needles of *N*-benzyl-1,1,3,3-tetramethylisoindoline **169** (2.88 g, 27%). Mp. 63-64 ^{o}C (lit.,[50] 63-64 ^{o}C). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.32 (s, 12 H, C*H*$_3$), 4.02 (s, 2 H, C*H*$_2$), 7.17 (dd, 2 H, Ar-*H)* 7.26 (dd, 2 H, Ar-*H*), 7.24-7.36 (m, 3 H, Ar-*H*), 7.51 (dd, 2 H, Ar-*H*). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 28.4 (*C*H_3), 46.4 (*C*H_2), 65.4 (*C*CH_3), 121.3 (Ar-*C*), 126.35 (Ar-*C*), 127.0 (Ar-*C*), 127.7 (Ar-*C*), 128.2 (Ar-*C*), 143.4 (Ar-*C*), 147.8 (Ar-*C*).

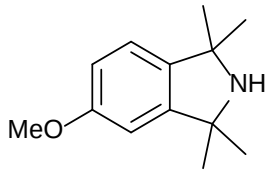
2.4.3 Synthesis of 5-Bromo-1,1,3,3-tetramethylisoindoline 66



A solution of *N*-benzyl-1,1,3,3-tetramethylisoindoline **169** (6 g, 22.7 mmol, 1 equiv.) in DCM (60 mL) was placed under Ar and cooled to 0 ^{o}C. To that was added a solution of bromine (2.43 mL, 47.5 mmol, 2.1 equiv.) in DCM (10 mL) dropwise, followed immediately by the addition of anhydrous aluminium trichloride ($AlCl_3$) powder (10.6 g, 79.5 mmol, 3.5 equiv.). The reaction was stirred for 1 hr at 0 ^{o}C and the resulting mixture poured onto ice and stirred vigorously for a further 10 minutes. The solution was then basified to or above pH 12 with aqueous NaOH (10 M) solution and then extracted with DCM. The combined DCM extracts were washed with brine solution and concentrated under reduced pressure to give golden oil. This oil was taken up in methanol and sodium bicarbonate (200 mg) added. A solution of aqueous hydrogen peroxide (30%) was then added dropwise until the observed effervescence ceased, and ensuring that some sodium bicarbonate remained. A solution of H_2SO_4 (2 M) was cautiously added and the resulting solution extracted with DCM (20 mL x 3). The combined DCM extracts were extracted further with H_2SO_4 (2 M, 20 mL x 4). The combined acidic layers were washed with DCM and

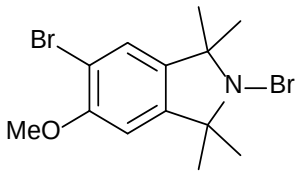
basified at 0 ^{o}C to pH 12. The resulting basic aqueous layer was extracted with DCM and the combined DCM layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 5-bromo-1,1,3,3-tetramethylisoindoline **66** as a clear oil which rapidly solidified upon standing in air (5.19 g, 90%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.43 (s, 6 H, CH_3), 1.45 (s, 6 H, CH_3), 6.98 (d, 1 H, Ar-*H*), 7.23 (d, 1 H, Ar-*H*), 7.34 (dd, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.7 (*C*H_3), 62.5 (*C*CH_3), 62.6 (*C*CH_3), 121.2 (Ar-*C*), 123.1 (Ar-*C*), 124.7 (Ar-*C*), 130 (Ar-*C*), 147.7 (Ar-*C*), 151.2 (Ar-*C*). The obtained NMR data was consistent with that previously reported in the literature.[3]

2.4.4 Synthesis of 5-Methoxy-1,1,3,3-tetramethylisoindoline 170



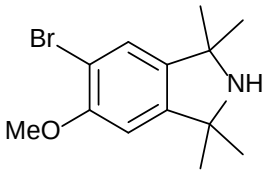
CuI (56.25 mg, 0.2 equiv.) was added to a solution of 5-bromo-1,1,3,3-tetramethylisoindoline **66** (500 mg, 1.967 mmol, 1 equiv.) in DMF (3 mL) and NaOMe (5 M in MeOH, 12 mL) under Ar. The reaction mixture was refluxed for 15 h and allowed to return to RT. It was then diluted with H_2O and extracted with Et_2O. The combined Et_2O (40 mL x 4) extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting solid residue was purified by silica column chromatography ($CHCl_3$/EtOH, 10:0.5) to give 5-methoxy-1,1,3,3-tetramethylisoindoline **170** as a pale white oil-solid (343.3 mg, 85%). Mp. 57-58 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.45 (s, 6 H, CH_3), 1.47 (s, 6 H, CH_3), 3.87 (s, 3 H, OCH_3), 6.65-7.39 (m, 3 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.9 (C-*C*H_3), 32.0 (C-*C*H_3), 55.4 (O*C*H_3), 62.5 (*C*CH_3), 62.8 (*C*CH_3), 112.8 (Ar-*C*), 123.3 (Ar-*C*), 125.7 (Ar-*C*), 140.0 (Ar-*C*), 147.2 (Ar-*C*), 157.4 (Ar-*C*). HRMS (ES): *m*/*z* (%) = 206.1574 (100) $[M + H]^{+}$, calcd. for $C_{13}H_{19}NO$: 205.1467; found 105.1470. ATR-FTIR: υ_{max}= 3415 (s, N-H), 1154 (s, C-N), 1042 (C-O) cm^{-1}.

2.4.5 Synthesis of 2,5-Dibromo-6-methoxy-1,1,3,3-tetramethylisoindoline 171



A solution of 5-methoxy-1,1,3,3-tetramethylisoindoline **170** (1.50 g, 731 μmol, 1 equiv.) in DCM (25 mL) under Ar was cooled to 0°C. A solution of bromine (942 μL, 1.83 mmol, 2.5 equiv.) in DCM (10 mL) was added dropwise followed by addition of anhydrous aluminium trichloride (3.48 g, 2.56 mmol, 3.5 equiv.). The reaction mixture was stirred for 1 hr at 0 °C, then poured onto ice (40 mL) and stirred vigorously for further 20 min. The solution was then basified to or above pH 12 with aqueous NaOH (10 M) solution and stirred for 10 min. The mixture was extracted with DCM (50 mL x 4) and the combined DCM extracts were washed with brine (50 mL), and the solvent removed under reduced pressure to give light yellow oil. The oil was triturated with methanol to give 2,5-dibromo-6-methoxy-1,1,3,3-tetramethylisoindoline **171** as a yellow solid (2.307 g, 87%). Mp. 97-98 °C. HPLC purity (93%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.41 (s, 6 H, C*H*$_3$), 1.44 (s, 6 H, C*H*$_3$), 3.91 (s, 3 H, OC*H*$_3$), 6.66 (s, 1 H, Ar-*H*), 7.3 (s, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 28.1 (C-*C*H$_3$), 28.3 (C-*C*H$_3$), 56.5 (O*C*H$_3$), 69.2 (*C*-CH$_3$), 69.7 (*C*-CH$_3$), 105.4 (Ar-*C*), 110.6 (Ar-*C*), 126.6 (Ar-*C*), 137.7 (Ar-*C*), 144.8 (Ar-*C*), 155.3 (Ar-*C*). ATR-FTIR: υ_{max}= 3000 (m, Ar C-H), 1232 (s, C-N), 1034 (C-O) cm^{-1}.

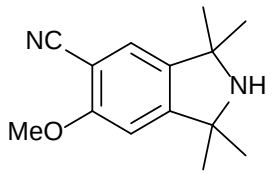
2.4.6 Synthesis of 5-Bromo-6-methoxy-1,1,3,3-tetramethylisoindoline 172



To a suspension of 2,5-dibromo-6-methoxy-1,1,3,3-tetramethylisoindoline **171** (900 mg, 2.479 mmol, 1 equiv.) and $NaHCO_3$ (208 mg, 2.479 mmol, 1 equiv.) in MeOH/DCM (10:5 mL) was added dropwise aqueous H_2O_2 (30%) until the observed effervescence ceased. The reaction mixture was stirred for 5 min followed

by the addition of NaOH (5 M). The resulting solution was extracted with DCM (40 mL x 4) and the combined DCM extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give 5-bromo-6-methoxy-1,1,3,3-tetramethylisoindoline **172** as a beige solid (688 mg, 98%). Mp. 59-60 ^oC. HPLC purity (>99%). ^1H NMR ($CDCl_3$, 600 MHz): δ = 1.42 (s, 6 H, C*H*$_3$), 1.45 (s, 6 H, C*H*$_3$), 3.91 (s, 3 H, OC*H*$_3$), 6.62 (s, 1 H, Ar-*H*), 7.26 (s, 1 H, Ar-*H*).). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.8 (C-*C*H$_3$), 32.0 (C-*C*H$_3$), 56.4 (O*C*H$_3$), 62.4 (*C*-CH$_3$), 62.8 (*C*-CH$_3$), 105.1 (Ar-*C*), 110.5 (Ar-*C*), 126.2 (Ar-*C*), 142.3 (Ar-*C*), 149.6 (Ar-*C*), 155.3 (Ar-*C*). HRMS (ES): *m*/*z* (%) = 184.0723/186.0723 (100) $[M + H]^+$; calcd. for $C_{13}H_{18}BrNO$: 283.0572; found 283.0574. ATR-FTIR: υ_{max}= 3307 (w, N-H), 2961 (m, Ar C-H), 1307 (s, C-N), 1038 (C-O) 699 (s, C-Br) cm^{-1}.

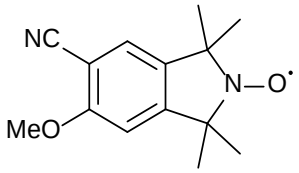
2.4.7 Synthesis of 5-Cyano-6-methoxy-1,1,3,3-tetramethylisoindoline 173



A Schlenk vessel that contained a mixture of 5-bromo-6-methoxy-1,1,3,3-tetramethylisoindoline **172** (2.76 g, 9.72 mmol, 1 equiv.), $K_4[Fe(CN)_6]$ (837 mg, 1.94 mmol, 0.2 equiv.), CuI (223 mg, 1.17 mmol, 0.12 equiv.) *N*-butylimidazole (2.5 mL, 19.45 mmol, 2 equiv.) in *o*-xylene (20 mL) was degassed and then refluxed at 180 ^oC for 3 d. The resulting mixture was allowed to return to RT before it was diluted with water and then extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (50 mL) and dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc) and recrystallized from cyclohexane to give 5-cyano-6-methoxy-1,1,3,3-tetramethylisoindoline **173** as an off-white solid (1.75 g, 78%). Mp. 138-139 ^oC. HPLC purity (>99%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (s, 6 H, C*H*$_3$), 1.45 (s, 6 H, C*H*$_3$), 1.73 (s, 1 H, N-H), 3.94 (s, 3 H, OC*H*$_3$), 6.66 (s, 1 H, Ar-*H*), 7.27 (s, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.6 (C-*C*H$_3$), 31.9 (C *C*H$_3$), 56.2 (O*C*H$_3$), 62.4 (*C*-CH$_3$), 63.2 (*C*-CH$_3$), 100.8 (C≡N), 104.5 (Ar-*C*), 117.0 (Ar-*C*), 126.8 (Ar-*C*), 141.4 (Ar-*C*), 156.2 (Ar-*C*), 161.5 (Ar-*C*). HRMS (ES):

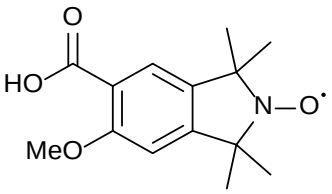
m/*z* (%) = 231.156 (100) $[M + H]^+$; calcd. for $C_{14}H_{18}N_2O$: 230.1419; found 230.1420. ATR-FTIR: υ_{max}= 3326 (w, N-H), 2968 (m, Ar C-H), 2221 (m, C≡N), 1155 (s, C-N), 1042 (C-O) cm^{-1}.

2.4.8 Synthesis of 5-Cyano-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 174



m-Chloroperoxybenzoic acid (1.78 g, 6.43 mmol, 1.3 equiv.) was added to a solution of 5-cyano-6-methoxy-1,1,3,3-tetramethylisoindoline **173** (1.14 g, 4.95 mmol, 1 equiv.) in DCM (100 mL) at 0 ^oC. The cooling bath was removed after 30 min and the reaction stirred at RT for a further 1.5 h. The DCM layer was washed with HCl (2 M), NaOH (5 M), and brine solutions (50 mL) and before being dried over anhydrous Na_2SO_4. The DCM was removed under reduced pressure and the solid residue obtained was recrystallized from EtOH to give bright yellow needles of 5-cyano-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **174** (1.09 g, 90%). Mp. 200-201 ^oC. HPLC purity (>99%). HRMS (ES): *m*/*z* (%) = 268.1230 (100) $[M + Na]^+$; calcd. for $C_{14}H_{17}N_2O_2^{\bullet}$: 245.1290; found 245.1286. ATR-FTIR: υ_{max}= 3048 (w, Ar C-H), 2231 (m, C≡N), 1472 (N-O), 1161 (s, C-N), 1041 (C-O) cm^{-1}.

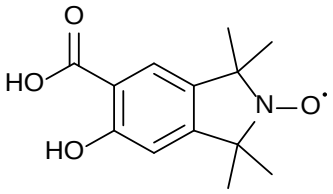
2.4.9 Synthesis of 5-Carboxy-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 175



A suspension of 5-cyano-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **174** (760 mg, 3.1 mmol, 1.00 equiv.) in NaOH (5 M, 10 mL)/EtOH (5 mL) was refluxed for 16 h. The reaction mixture was cooled to RT, then diluted with H_2O and washed with Et_2O (40 mL x 2). The Et_2O layer was discarded. The aqueous layer was cooled in ice bath and acidified with HCl (2 M) before it was extracted with Et_2O (50 mL x 4).

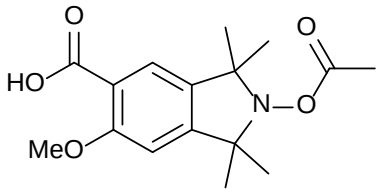
The combined Et_2O extracts were washed with brine (50 mL) and dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was recrystallized from H_2O/EtOH to give 5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **175** as yellow solid (729 mg, 89%). Mp. 244-245 ^oC (dec.). HPLC purity (>99%). HRMS (ES): *m*/*z* (%) = 287.1714 (100) $[M + Na]^+$, 303.0992 (15) $[M + K]^+$; calcd. for $C_{14}H_{18}NO_4^{\bullet}$: 264.1236; found 264.1241. ATR-FTIR: υ_{max}= 3400-2450 (m, br, OH), 2973 (m, Ar C-H), 1675 (s, C=O), 1360 (s, C-N), 1202 (C-O) cm^{-1}.

2.4.10 Synthesis of 5-Carcoxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl salicylic acid TMIO 137) via BBr_3 Demethylation of 175



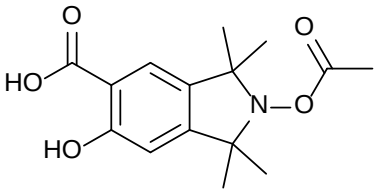
BBr_3 (1.9 mL, 1.89 mmol, 1 M solution in DCM, 2.5 equiv.) was added dropwise to a solution of 5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **175** (200 mg, 757 µmol, 1 equiv.) in DCM (15 mL) at -78 ^oC under Ar atmosphere. The reaction was allowed to return to RT while stirring for 18 h. H_2O was added to the resulting mixture to quench excess BBr_3 reagent. The crude product was extracted with EtOAc (50 mL x 4) and the combined EtOAc extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (DCM/MeOH, 6:0.4) and recrystallized from H_2O/EtOH to give 5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **137** as yellow crystals (76 mg, 40%). Mp. 207-208 ^oC (dec.). HPLC purity (>99%). HRMS (ES): *m*/*z* (%) = 152.1186 (45) $[M + 2H]^+$; calcd. for $C_{13}H_{16}NO_4^{\bullet}$: 250.1079; found 250.1071. ATR-FTIR: υ_{max}= 3400-2500 (m, br, OH), 2972 (m, Ar C-H), 1674 (s, C=O), 1201 (C-O) cm^{-1}.

2.4.11 Synthesis of 2-Acetoxy-5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindoline 185



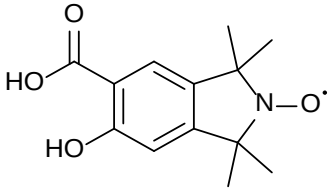
A reaction mixture of 5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **175** (660 mg, 2.5 mmol, 1 equiv.) and Pd/C (66 mg, 10%, 62.5 µmol, 0.025 equiv.) in THF (15 mL) was flushed with Ar for 10 min. Then, a balloon of H_2 was connected and the reaction mixture stirred for 15 min and then cooled in ice/H_2O bath. TEA (697 µL, 5 mmol, 2 equiv.) and then AcCl (355 µL, 5 mmol, 2 equiv.) were added dropwise and the resulting mixture was stirred for 30 min. The cooling bath was removed and stirring was continued for a further 1 h. The reaction mixture was filtered through Celite and concentrated *in vacuo*. The crude residue was stirred in aqueous MeOH (10 mL, 2 mL H_2O) for 1 h, then diluted with H_2O, and extracted with EtOAc (50 mL x 4). The EtOAc extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give compound 2-acetoxy-5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindoline **185** as a clear solid (738 mg, 96%). Although compound **185** was pure enough to be used subsequent step, it was further purified by silica gel flash column chromatography (EtOAc/$CHCl_3$, 2:1) and recrystallization from cyclohexane. Mp. 149-150 ^oC. HPLC purity (>99%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (d, 6 H, C*H*$_3$), 1.48 (d, 6 H, C*H*$_3$), 2.2 (s, 3 H, C=OC*H*$_3$), 4.1 (s, 3 H, OC*H*$_3$), 6.78 (s, 1 H, Ar-*H*), 7.98 (s, 1 H, Ar-*H*).), 10.81 (s, br, 1 H, CO_2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 19.2 (CO-*C*H_3) 25.0, 25.2 (C-*C*H_3), 28.6, 28.8 (C-*C*H_3), 57.0 (O*C*H_3), 67.9 (*C*-CH_3), 68.4 (*C*-CH_3), 105.0 (Ar-*C*), 117.4 (Ar-*C*), 127.5 (Ar-*C*), 138.1 (Ar-*C*), 151.7 (Ar-*C*), 158.3 (Ar-*C*), 165.2 (*C*O_2H), 171.2 (*C*=OCH_3). HRMS (ES): *m*/*z* (%) = 330.1401 (20) $[M + Na]^+$, 346.1140 (24) $[M + K]^+$; calcd. for $C_{16}H_{21}NO_5$: 307.1420; found 307.1418. ATR-FTIR: υ_{max}= 3267 (m, br, OH), 2973 (m, Ar C-H), 1772 (s, Ac C=O), 1709 (carboxylic acid C=O) 1194 (C-O) cm^{-1}.

2.4.12 Synthesis of 2-Acetoxy-5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindoline 186



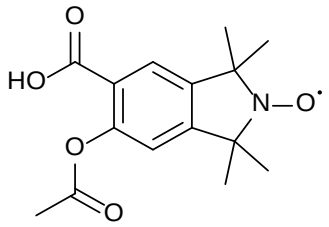
BBr_3 (1.4 mL, 1.4 mmol, 1 M solution in DCM, 2.5 equiv.) was added dropwise to a solution of 2-acetoxy-5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindoline **185** (170 mg, 559 µmol, 1 equiv.) in DCM (10 mL) at -78 ^{o}C under Ar atmosphere. The reaction was allowed to return to RT while stirring for 18 h. H_2O was added to the resulting mixture to quench excess BBr_3 reagent. The crude product was extracted with EtOAc (20 mL x 4) and the combined EtOAc extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (DCM/MeOH, 6:0.4) and recrystallized from cyclohexane to give 2-acetoxy-5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindoline **186** as a white solid (153 mg, 85%). Mp. 168-169 ^{o}C. HPLC purity (>98%).^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.4 (s, 6 H, C*H*$_3$), 1.48 (s, 6 H, C*H*$_3$), 2.2 (s, 3 H, C=OC*H*$_3$), 6.77 (s, 1 H, Ar-*H*), 7.64 (s, 1 H, Ar-*H*), 10.6 (s, br, 1 H, CO_2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 19.2 (CO-*C*H_3) 25, 25.2 (C-*C*H_3), 28.6, 28.8 (C-*C*H_3), 57.0 (O*C*H_3), 67.9 (*C*-CH_3), 68.4 (*C*-CH_3), 105.0 (Ar-*C*), 117.4 (Ar-*C*), 127.5 (Ar-*C*), 138.07 (Ar-*C*), 151.7 (Ar-*C*), 158.3 (Ar-*C*), 165.2 (*C*O_2H), 171.2 (*C*=OCH_3). HRMS (ES): *m*/*z* (%) = 294.2269 (15) $[M + H]^+$, calcd. for $C_{15}H_{19}NO_5$: 293.1263; found 293.1272. ATR-FTIR: υ_{max}= 3095 (m, br, OH), 2973 (m, Ar C-H), 1737 (s, Ac C=O), 1677 (carboxylic acid C=O), 1160 (C-O) cm^{-1}.

2.4.13 Synthesis of 5-Carboxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (salicylic acid TMIO 137) via Hydrolysis of 186



A suspension of 2-acetoxy-5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindoline **186** (136 mg, 464 µmol, 1 equiv.) in H_2O/MeOH (2 mL/2 mL) was cooled to 0 ^oC. LiOH (56 mg, 2.3 mmol, 5 equiv.) was added and the reaction mixture stirred overnight while allowed to warm to RT. The resulting solution was washed with Et_2O and the Et_2O layer discarded. The aqueous layer was cooled in ice bath, acidified with HCl (2 M) and then extracted with Et_2O (30 mL x 4). The combined Et_2O extracts were stirred over PbO_2 (28 mg, 116 µmol, 0.25 equiv.) for 20 min, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude residue was recrystallized from H_2O/EtOH to give 5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **137** as yellow crystals (107 mg, 92%). Mp. 207-208 ^oC (dec.). HPLC purity (>99%). HRMS (ES): *m*/*z* (%) = 152.1186 (45) $[M + 2H]^+$; calcd. for $C_{13}H_{16}NO_4^{\bullet}$: 250.1079; found 250.1071. ATR-FTIR: υ_{max}= 3400-2500 (m, br, OH), 2972 (m, Ar C-H), 1674 (s, C=O), 1201 (C-O) cm^{-1}.

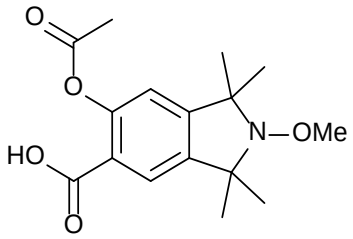
2.4.14 Synthesis of 6-Acetoxy-5-caarboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (Aspirin TMIO 138)



A solution of 5-carboxy-6-hydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **137** (89 mg, 354.4 µmol, 1 equiv.) in THF under Ar was cooled to 0^oC. TEA (99 µL, 709 µmol, 2 equiv.) and then AcCl (50.4 µL, 709 µmol, 2 equiv.) were added dropwise and the resulting mixture stirred for 3 h while allowing to return to RT. Water was added to the reaction mixture and it was then extracted with DCM (30 mL x 3). The DCM extracts were washed with HCl (1 M, 15 mL), brine (20 mL) and dried over anhydrous Na_2SO_4. The combined DCM was removed under reduced pressure and the crude residue obtained was recrystallized from H_2O/EtOH to give 6-acetoxy-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **138** as a yellow solid (94 mg, 91%). Mp. 206-207 ^oC. HPLC purity (>99%). HRMS (ES): *m*/*z* (%) = 293.1221 (100) $[M + H]^+$. calcd. for $C_{15}H_{18}NO_5^{\bullet}$: 292.1185; found 292.1183. ATR-FTIR: υ_{max}= 3400-

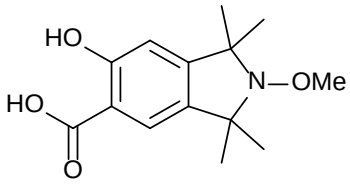
2600 (m, br, OH), 2972 (m, Ar C-H), 1765 (s, Ac C=O), 1695 (carboxylic acid C=O) 1184 (C-O) cm^{-1}.

2.4.15 Synthesis of 6-Acetoxy-5-carboxy-2-methoxy-1,1,3,3-tetramethylisoindoline (Aspirin TMIO methoxyamine derivative) 193



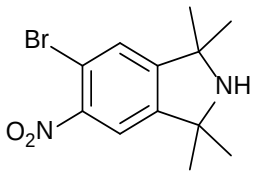
A solution of H_2O_2 (186 µL, 889 µmol, 2 equiv, 30%) was added dropwise (over 10 min) to a solution of 6-acetoxy-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **138** (130 mg, 445 µmol, 1 equiv.) and $FeSO_4.7H_2O$ (247 mg, 889 µmol, 2 equiv.) in DMSO (8 mL). The resulting reaction mixture was stirred for 1 h and then diluted with H_2O. The aqueous layer was extracted with Et_2O (30 mL x 4) and the combined Et_2O extracts were washed with dilute aqueous HCl (2 M, 20 mL), brine (20 mL), and then dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the crude residue purified by short silica gel column chromatography (EtOAc) and recrystallization (cyclohexane/EtOAc) to give 6-acetoxy-5-carboxy-2-methoxy-1,1,3,3-tetramethylisoindoline **193** as white solid (88 mg, 64%). Mp. 215-217 ^{o}C (dec.). HPLC purity (>99%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.44 (br, s, 12 H, C*H*$_3$), 2.34 (s, 3 H, OAc), 3.77 (s, 3 H, OC*H*$_3$), 6.85 (s, 1 H, Ar-*H*), 7.82 (s, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 21.1 (C=O-*C*H$_3$), 25.2 (br, C-*C*H$_3$), 29.7 (br, C-*C*H$_3$), 65.6 (O*C*H$_3$), 66.9 (*C*-CH$_3$), 67.3 (*C*-CH$_3$), 117.2 (Ar-*C*), 121.0 (Ar-*C*), 125.8 (Ar-*C*), 143.2 (Ar-*C*), 151.0 (Ar-*C*), 152.9 (Ar-*C*), 169.4 (*C*O$_2$H), 169.9 (*C*=OCH$_3$). HRMS (ES): *m*/*z* (%) = 308.1453 (25) $[M + H]^+$; calcd. for $C_{16}H_{21}NO_5$: 307.1420; found 307.1417. ATR-FTIR: υ_{max}= 3400-2600 (m, br, OH), 2973 (m, Ar C-H), 1762 (s, Ac C=O), 1695 (carboxylic acid C=O) 1193 (C-O) cm^{-1}.

2.4.16 Synthesis of 5-Carboxy-6-hydroxy-2-methoxy-1,1,3,3-tetramethylisoindoline (Salicylic acid TMIO methoxyamine derivative) 194



Dilute sodium hydroxide solution (2 mL, 2 M) was added to a solution of 6-acetoxy-5-carboxy-2-methoxy-1,1,3,3-tetramethylisoindoline **193** (50 mg, 163 µmol, 1 equiv.) in H_2O/MeOH (2 mL/2 mL) and the reaction mixture stirred at RT for 3 h. The resulting solution was washed with Et_2O and the Et_2O layer discarded. The aqueous layer was cooled in ice bath, acidified with HCl (2 M) and then extracted with Et_2O (20 mL x 4). The combined Et_2O extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude residue was recrystallized from cyclohexane/EtOAc to give 5-carboxy-6-hydroxy-2-methoxy-1,1,3,3-tetramethylisoindoline **194** as white solid (41.4 mg, 96%). Mp. 210-212 ^{o}C (dec.). HPLC purity (>99%). HRMS (ES): *m*/*z* (%) = 266.1530 (100) $[M + H]^+$; calcd. for $C_{14}H_{19}NO_4$: 265.3090; found 265.3090. ATR-FTIR: υ_{max}= 3400-2500 (m, br, OH), 2991 (m, Ar C-H), 1656 (s, C=O), 1209 (C-O) cm^{-1}.

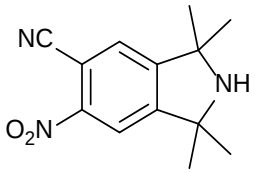
2.4.17 Synthesis of 5-Bromo-1,1,3,3-tetramethyl-6-nitroisoindoline 67



5-Bromo-1,1,3,3-tetramethylisoindoline **66** (1.9 g, 7.48 mmol) was dissolved in H_2SO_4 (15 mL, 98%) and cooled to 0 °C. Nitric acid (3.75 mL, 70%) was added dropwise and the reaction mixture was stirred at 0 °C for 1 h. The cooling bath was removed and stirring was continued for 4 h. The reaction mixture was cooled to 0 °C, diluted by careful addition ice/H_2O and then basified with NaOH solution (10 M). The reaction mixture was extracted with Et_2O (60 mL x 4) and the combined organic extracts were washed with brine (50 mL), dried with anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The residue was purified by filtration

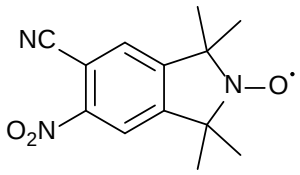
through a short silica gel column (EtOAc) and recrystallized from hexane/EtOAc (5:1) to give 5-bromo-1,1,3,3-tetramethyl-6-nitroisoindoline **67** as yellow crystals (1.23 g, 55%). Mp. 135–137 °C, (lit.,[51] 136–138 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.47 (s, 12 H, C*H*$_3$), 1.6 (br. s, 1 H, N*H*), 7.42 (s, 1 H, Ar-*H*), 7.58 (s, 1 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.6, 31.6 (C-*C*H_3), 62.8, 62.9 (*C*-CH_3), 113.3 (Ar-*C*), 119.2, 128.1 (Ar-*C*), 149.9 (Ar-*C*), 154.9 (Ar-*C*) ppm.

2.4.18 Synthesis of 5-Cyano-1,1,3,3-tetramethyl-6-nitroisoindoline 187



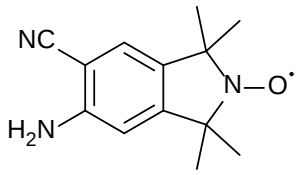
A Schlenk vessel that contained a mixture of 5-bromo-1,1,3,3-tetramethyl-6-nitroisoindoline **67** (1.3 g, 4.35 mmol, 1.00 equiv.), $K_4[Fe(CN)_6]$ (329 mg, 870 µmol, 0.2 equiv.), CuI (83 mg, 435 µmol, 0.1 equiv.) and *N*-butylimidazole (1.17 mL, 8.7 mmol, 2 equiv.) in toluene (15 mL) was degassed and then refluxed at 150 ^{o}C for 2 d. The resulting mixture was allowed to return to RT before it was diluted with water and then extracted with EtOAc (50 mL x 4). The combined organic extracts were washed with brine (40 mL) and dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc) and recrystallized from hexane/EtOAc give 5-cyano-1,1,3,3-tetramethyl-6-nitroisoindoline **187** as a beige powder (726 mg, 68%). Mp. 159–160 °C, (lit.,[51]158–160 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.5 (s, 6 H, C*H*$_3$), 1.51 (s, 6 H, C*H*$_3$), 1.71 (br, s, 1 H, N*H*), 7.6 (s, 1 H, Ar-*H*), 8.04 (s, 1 H, Ar-*H*) ppm. ^{13}C NMR (CDCl3, 100 MHz): δ = 31.5, 31.6 (*C*H_3), 63.2, 63.3 (*C*CH_3), 107.4, (*C*N), 115.4 (Ar- *C*), 129.4 (Ar-*C*), 148.7 (Ar-*C*), 155.8 (Ar-*C*), 115.9 (Ar-*C*) ppm. ATR-FTIR: υ_{max}= 3042 (w, N-H), 2980 (m, ArC-H), 2235 (m, C≡N), 1526 (s, NO_2), 1337 (s, C-N), 1179 (s, C-O) cm^{-1}.

2.4.19 Synthesis of 5-Cyano-1,1,3,3-tetramethyl-6-nitroisoindolin-2-yloxyl 188



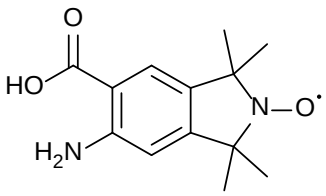
A solution of 5-cyano-1,1,3,3-tetramethyl-6-nitroisoindoline **187** (500 mg, 2.04 mmol, 1.00 equiv.) in DCM (100 mL) was treated with *m*CPBA (595 mg, 2.65 mmol, 77%, 1.20 equiv.) and stirred for 2 h. The reaction mixture was washed with NaOH (2 M, 20 mL x 3) and brine (40 mL), then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was recrystallized from MeCN to give 5-cyano-1,1,3,3-tetramethyl-6-nitroisoindolin-2-yloxyl **188** as orange needles (494 mg, 93%). Mp. 230-231 °C (dec), (lit.,[51] >230 °C, dec.). ATR-FTIR: υ_{max}= 2981 (m, ArC-H), 2233 (m, C≡N), 1530 (s, NO_2), 1340 (s, C-N), 1163 (s, C-O) cm^{-1}.

2.4.20 Synthesis of 5-Amino-6-cyano-1,1,3,3-tetramethylisoindolin-2-yloxyl 189



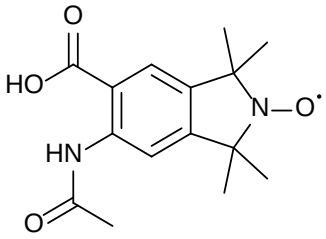
A suspension of $SnCl_2 \cdot 2H_2O$ (403 mg, 1.79 mmol, 3.00 equiv.) in conc. HCl (2 mL) was cooled to 0 °C. 5-Cyano-1,1,3,3-tetramethyl- 6-nitroisoindolin-2-yloxyl **188** (155 mg, 596 µmol, 1 equiv.) was added and the mixture was stirred at 0 °C for 10 min. The cooling bath was removed and stirring was continued for an additional 3 h. The reaction mixture was cooled to 0 °C and basified by careful addition of NaOH (5 M). The mixture was then extracted with DCM (30 mL x 4) and the combined DCM extracts were washed with brine (30 mL), dried with Na_2SO_4 and the solvent evaporated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane/EtOAc, 1:1) to give 5-amino-6-cyano-1,1,3,3-tetramethylisoindolin-2-yloxyl **189** as a pale beige solid (105 mg, 69%). Mp. 199–200 °C (lit.,[51] 198-200 °C).

2.4.21 Synthesis of 6-Amino-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 190



A suspension of 5-amino-6-cyano-1,1,3,3-tetramethylisoindolin-2-yloxyl **189** (100 mg, 434 µmol, 1.00 equiv.) in 5 M NaOH/EtOH (3 mL/2 mL) was refluxed for 16 h. The reaction mixture was cooled to RT and diluted with H_2O. It was then washed with Et_2O (10 mL x 2) and the Et_2O layer was discarded. The aqueous layer was cooled in an ice bath, acidified with HCl (2 M) and extracted with Et_2O (20 mL x 4). The combined Et_2O extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was recrystallized from H_2O/MeCN to give 6-amino-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **190** as yellow, voluminous needles (81, 75%). Mp. 230 °C (dec) (lit.,[51] >230 °C, dec.). ATR-FTIR: υ_{max}= 3472, 3359 (m, N-H_2), 2928 (br, O-H), 1666 (s, C=O), 1282 (s, C-N), 1228 (C-O) cm^{-1}.

2.4.22 Synthesis of 6-Acetamido-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 139



A solution of 6-amino-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **190** (90 mg, 361 µmol, 1 equiv.) in THF (10 mL), and under Ar, was cooled to 0 °C. TEA (106 µL, 758 µmol, 2.1 equiv.), then AcCl (51 µL, 722 µmol, 2 equiv.) were added dropwise and the resulting mixture stirred for 3 h while allowing it to return to RT. Water was added to the reaction mixture, and then extracted with DCM (20 mL x 4). The DCM extracts were washed with HCl (1 M, 15 mL), brine and dried over anhydrous Na_2SO_4. The combined DCM was removed under reduced pressure and

the crude residue obtained was recrystallized from H_2O/MeCN to give 6-acetamido-5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **139** as a yellow solid (93 mg, 88%). Mp. 231-232 ^oC (dec.). HPLC purity (>98%). HRMS (ES): *m*/*z* (%) = 292.1413 (30) $[M + H]^+$, 314.1232 (70) $[M + Na]^+$; calcd. for $C_{15}H_{19}N_2O_4^{\bullet}$: 291.1345; found 291.1343. ATR-FTIR: υ_{max}= 33400-2600 (br, O-H), 3262 (w, N-H), 2988 (w, Ar C-H), 1681(s, CO_2H C=O), 1590 (s, Ac C=O), 1251 (s, C-N), 1198 (C-O) cm^{-1}.

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Chapter 3: Nitroxide-NSAID Conjugates: Design, Synthesis and Preliminary Biological Evaluation

3.1 Background

In the preceding chapter (**Chapter 2**), the pharmacophore hybridization strategy was employed in the design of merged nitroxide-aspirin based hybrids as potential dual action therapeutics for inflammatory and oxidative stress related diseases. Preliminary evaluation of the antioxidant and anti-inflammatory properties of the merged hybrids (**Chapter 2**) revealed their promising therapeutic potential. In this chapter, this hybridization approach is further extended by efforts to combine nitroxides with other NSAIDs to generate a series of novel nitroxide-NSAID conjugates. In addition to the potential therapeutic outcome, combining a number of nitroxide compounds (with various ring classes and substitutions) with NSAIDs through ester, amide and amine bonds provides an attractive way to investigate structure-activity relationships. **Figure 3.1** below shows the representative cleavable and non-cleavable target compounds in this chapter.

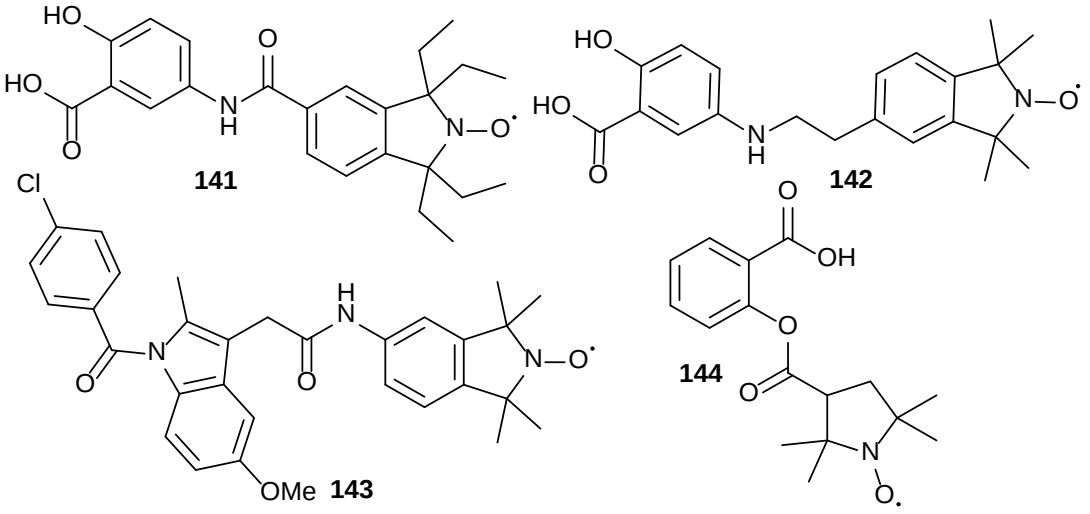
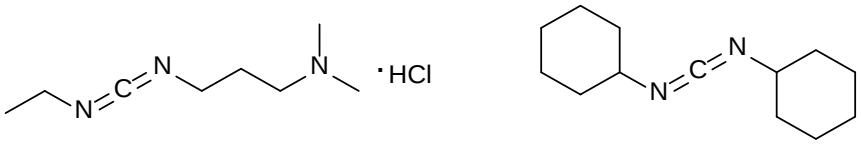


Figure 3.1. Representative target NSAID-nitroxide conjugates.

The NSAID pharmacophores to be linked with various nitroxide compounds include aspirin, salicylic acid, indomethacin, 5-aminosalicylic acid and 2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic acid. The synthesis of target compounds was carried out primarily by carbodiimide-mediated coupling reactions.

3.2 Carbodiimide Chemistry

Carbodiimides are unsaturated compounds that have a heterocumulene (allene type) structure with two reactive centres (**Figure 3.2**).[1-3] The central carbon atom is electrophilic and so reacts with nucleophiles when one of the nucleophilic terminal nitrogen atoms is activated by an electrophile such as a proton donor. The carbodiimide coupling reaction is widely used in the synthesis of peptides.

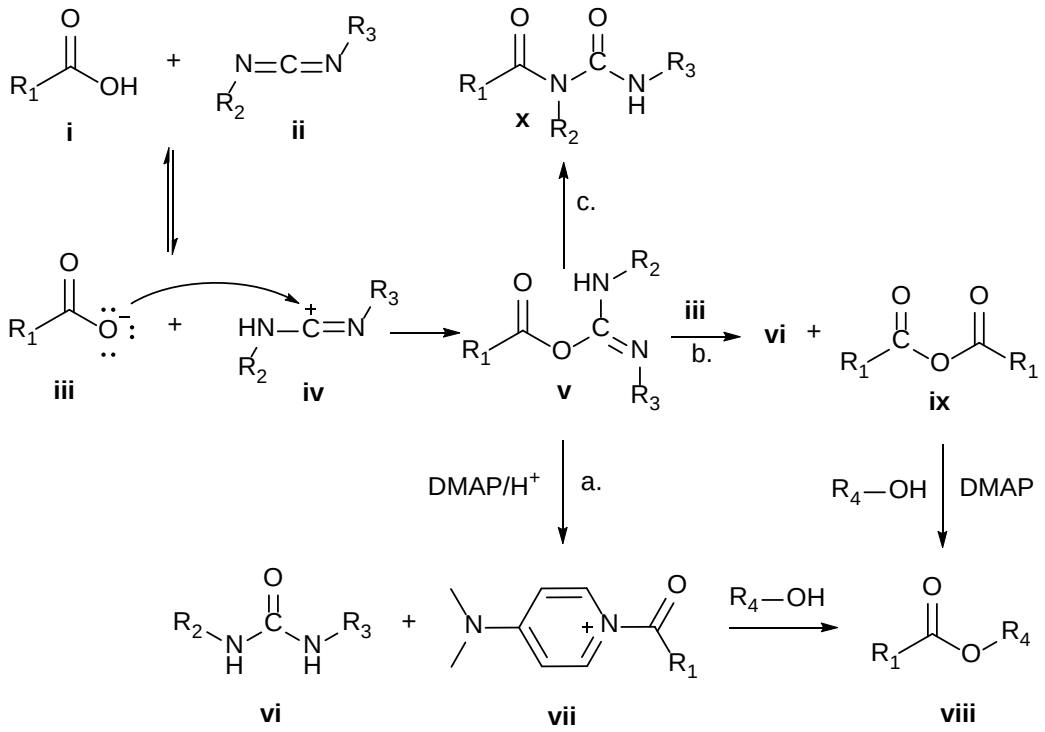


202 **203**

Figure 3.2. Common carbodiimides: EDC (**202**) and DCC (**203**).

The most common carbodiimides used in the synthesis of amides and esters are the commercially available *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride **202** (EDC) and *N*',*N*'-dicyclohexylcarbodiimide **203** (DCC). These reagents work by activating carboxylic acids (**i**, **Scheme 3.1**) through the formation of the unstable *O*-acylisourea intermediate (**v**).[4-6] The first step involves protonation of the nucleophilic nitrogen atom of the carbodiimide (**ii**) by the carboxylic acid to give a carbocation (**iv**). The resulting carboxylate (**iii**) then attacks the carbocation (**iv**) to form the *O*-acylisourea intermediate (**v**). The formation of amides or esters from the *O*-acylisourea intermediate (**v**) proceeds through two pathways (**a.** and **b.**). In the presence of DMAP, the *O*-acylisourea (**v**) can directly react with a nucleophile (alcohol or amine) to give the desired product (amide or ester) along with the urea by-product (**vi**). The *O*-acylisourea (**v**) can also react with a preformed nucleophilic carboxylate (**iii**) to form the anhydride (**ix**), which in turn reacts with an amine or alcohol to give the desired product. The urea by-product (**vi**) from DCC is usually insoluble and is removed from the reaction mixture by filtration. EDC on the other

hand gives a water-soluble urea by-product that is removed by an aqueous wash during the workup.



Scheme 3.1. Proposed mechanism for carbodiimide-mediated synthesis of esters from carboxylic acids and alcohols.

In carbodiimide-mediated ester or amide synthesis, the reaction is usually accompanied by the formation of an unreactive *N*-acylurea side product (**x**). This occurs when the *O*-acylisourea intermediate (**v**) undergoes an intramolecular O→N acyl rearrangement. However, DMAP as a hyperacylation catalyst (acylation catalyst) suppresses the formation of the *N*-acylurea side product (**x**). These coupling reactions are mostly carried out in dichloromethane.

3.3 Results and Discussion

The NSAIDs which are intended to be linked with nitroxides include salicylic acid **135**, aspirin **136**, 5-amino salicylic acid **149** and indomethacin **151**. **Figure 3.3** below shows the structures of the precursor nitroxide compounds used herein. As mentioned earlier in the introductory chapter, cyclic nitroxides such as TEMPOL **102** are more stable and have been used extensively as potent antioxidants. Whereas the

pyrrolidine and piperidines nitroxides are commercially available, the isoindoline nitroxides were synthesized as detailed in the subsequent section (except for compound **175**, synthesized in **Chapter 2**).

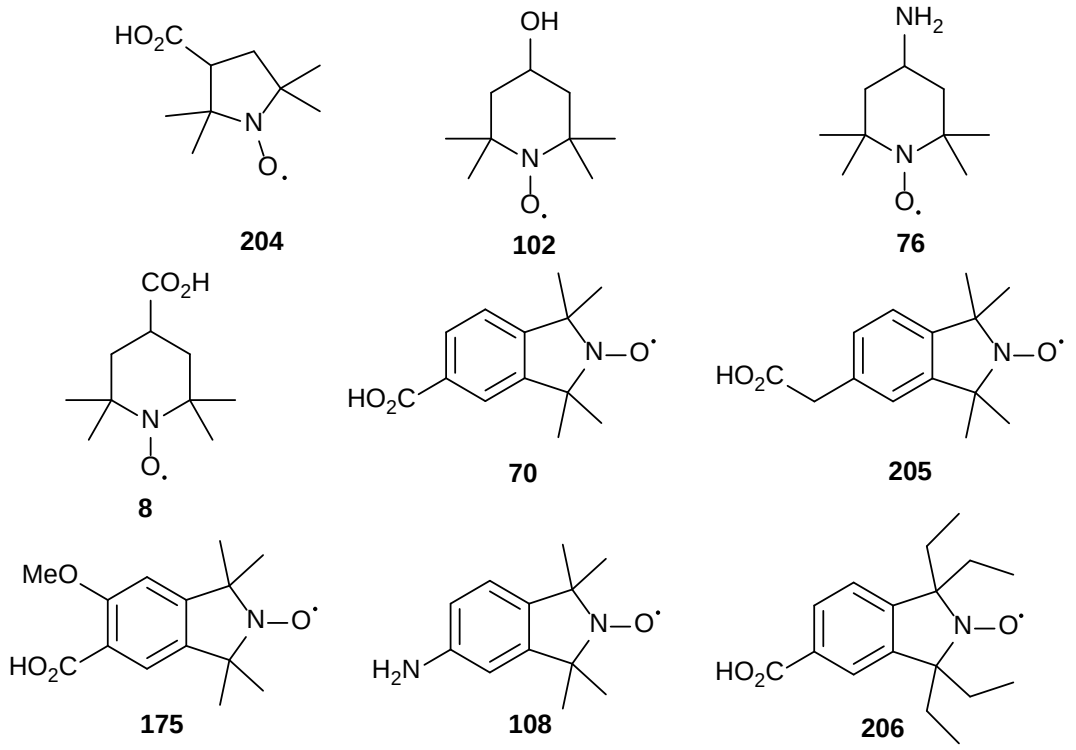
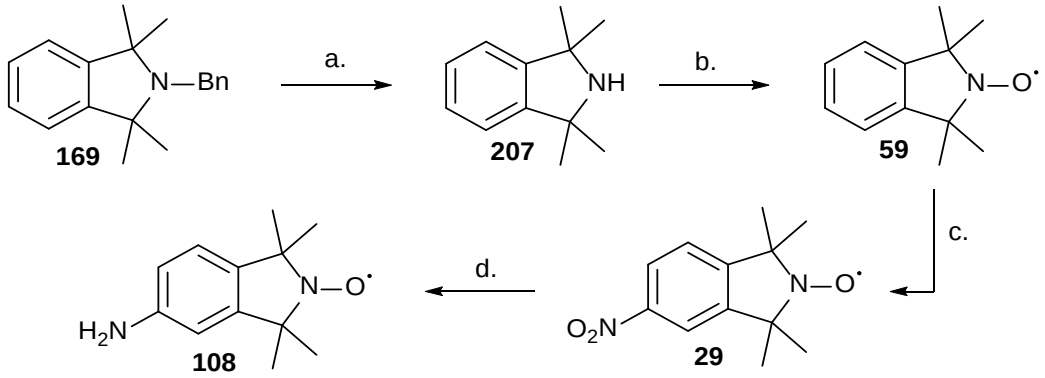


Figure 3.3. Various nitroxide compounds linked to NSAIDs.

3.3.1 Synthesis of 5-Amino-1,1,3,3-tetramethylisoindolin-2-yloxyl 108

The 5-amino-TMIO **108** was prepared from the precursor **169** following literature procedures with a slight modification (**Scheme 3.2**).[7,8] Compound **169** was synthesized by exhaustive methylation *N*-benzylphthalimide **168** with freshly prepared methyl Grignard reagent as detailed in **Chapter 2** (**Section 2.33**). Palladium-catalyzed hydrogenation of compound **169** was carried out in a Parr Hydrogenator to give the secondary amine derivative **207** in 96% yield. Subsequent oxidation of compound **207** to the nitroxide derivative **59** was performed with *m*CPBA. Compound **59** was obtained almost quantitatively as a bright yellow solid.



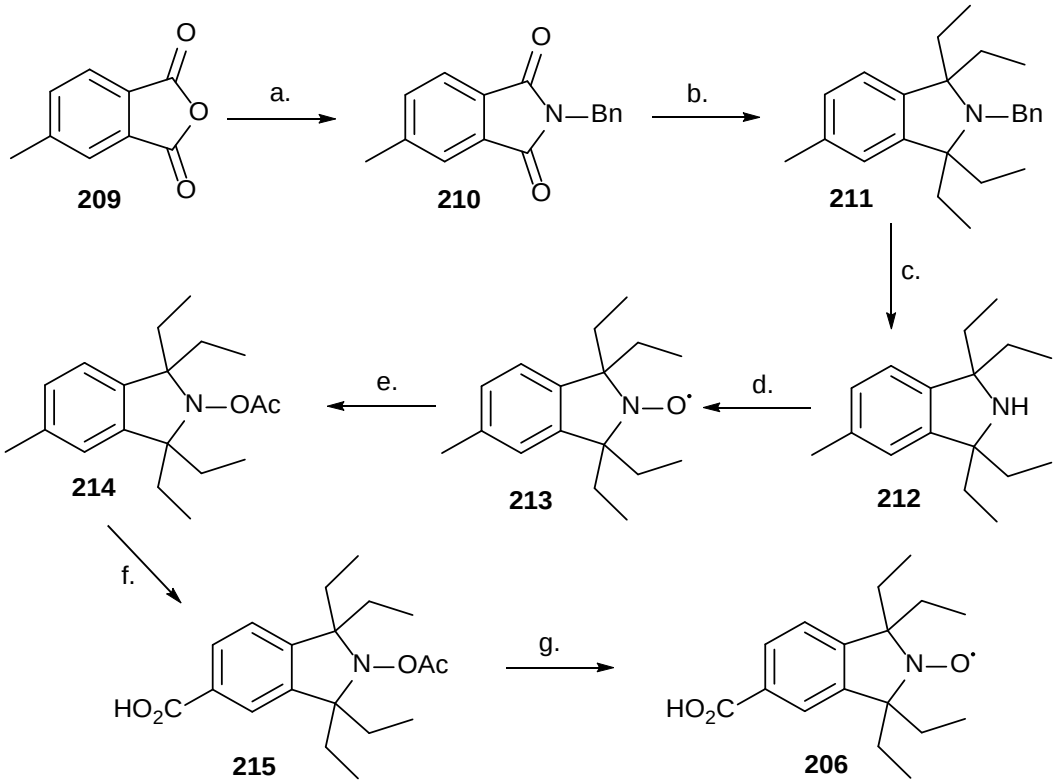
Scheme 3.2. Synthesis of 5-amino TMIO **108**. Reagents and conditions: a. H_2, Pd/C, 4 h at 50 psi, 96%; b. *m*CPBA, DCM, 0 ^oC to RT, 3 h, 94%; c. HNO_3, H_2SO_4, 0 ^oC, 5 h, 81%; d. H_2, Pd/C, 4 h at 50 psi, MeOH, then PbO_2, 88%.

The nitro derivative **29** was prepared by nitration of compound **59** using concentrated sulfuric acid and nitric acid to give a dark brown solution of what was presumed to be the oxoammonium salt (the oxoammonium salts obtained when isoindoline nitroxides disproportionate are characteristically reddish brown in solution). Although the crude nitro product obtained was of sufficient purity, it was further purified by recrystallization from ethanol to give bright orange crystals of **29**. For optimum yield in the next step, recrystallization of the crude nitro compound **29** was necessary otherwise, a decrease in the yield of the amino nitroxide **108** from 88% to 52% was observed when the crude nitro compound **29** was subjected to a palladium-catalyzed hydrogenolysis.

The crude product obtained after hydrogenolysis of **29**, was a mixture of the desired amino nitroxide **108** and the hydroxylamine intermediate. Though the hydroxylamine intermediate readily oxidizes to the nitroxide derivative **108** upon exposure to air, the crude mixture was stirred for 20 min over 0.025% mole of lead (II) oxide to ensure complete oxidation. Copper (II) acetate has equally been reported to facilitate the oxidation of the hydroxylamine.[9] Compound **108** was obtained as a yellow crystalline solid after recrystallization from ethanol. All characterization data obtained for compound **108** and its precursors were in agreement with that previously reported in the literature.[7,8]

3.3.2 Synthesis of 5-Carboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl 206 (CTEIO)

The tetraethylisoindoline carboxylic acid **206** was synthesized according to literature procedures as outlined in **Scheme 3.3** below.[10] The *N*-benzylphthalimide **210** was obtained quantitatively by refluxing commercially available 4-methylphthalic anhydride **209** and benzylamine in acetic acid for 1 h.



Scheme 3.3. Synthesis of CTEIO **206**. Reagents and conditions: $BnNH_2$, AcOH, reflux, 1 h, 98%; b. EtMgI, PhMe, reflux, 4 h, 32%; c. H_2 (50 psi), Pd/C, AcOH, RT, 4 h, 96%; d. *m*CPBA, DCM, RT, 3 h, 84%; e. AcCl, TEA, 0 ^oC, 30 min. to RT, 1 h, 91%; f. $KMnO_4$, *t*-BuOH, $MgSO_4$, 70 ^oC, 1 d, 60%; g. NaOH, overnight, 83%.

The phthalimide **210** was tetraalkylated with freshly prepared ethyl magnesium Grignard reagent by refluxing in anhydrous toluene under an argon atmosphere for 4 h. Purification of the crude tetraalkylated product involved filtration through Celite and basic alumina to afford pure **211** as clear oil in 32% yield. Compound **211** was then debenzylated to the corresponding secondary amine **212** by hydrogenolysis with

Pd/C in acetic acid. The secondary amine **212**, obtained as yellow oil, was oxidized with *m*CPBA to the nitroxide derivative **213** in 84% yield.

The key step in the synthesis of CTEIO **206** is the oxidation of the aryl methyl group with potassium permanganate to the corresponding carboxylic acid. Permanganate oxidation is an attractive and convenient method for regio-selective oxidation of species bearing mononuclear aromatic hydrocarbons side chains, such as toluene, to the corresponding carboxylic acids.[11,12]

To successfully carry out this transformation, protection of the nitroxide moiety was required due to its reported decomposition under refluxing permanganate oxidation conditions.[10] Thus, compound **213** was protected with an acetyl protecting group by first reducing it to the hydroxylamine derivative through hydrogenolysis, followed by *in situ* reaction with acetyl chloride in the presence of triethylamine to give the *N*-acetoxy protected compound **214**. The singlets at 2.52 and 2.75 ppm, each integrating for 3 protons in the ^{1}H NMR spectrum of **214**, were assigned to the *N*-acetoxy and the aryl methyl groups respectively.

Oxidation of the aryl methyl group of **214** was accomplished by allowing it to react for 24 hours with refluxing aqueous potassium solution in the presence of magnesium sulfate. As permanganate, when heated and/or exposed to air, quickly becomes oxidized to the black manganese dioxide residue (MnO_2), the oxidation reaction was carried out under an argon atmosphere and the permanganate reagent was added portionwise over the 24 hours. The *N*-acetoxy carboxylic acid **215** was obtained as a white solid (60%) after purification by column chromatography and recrystallization from hexane/ethyl acetate.

The target nitroxide **206** was readily obtained after deprotection of the nitroxide group by basic hydrolysis of **215** with lithium hydroxide. Compound **206** was obtained as a yellow crystalline solid after recrystallization from acetonitrile (84%). All characterization data obtained for compound **206** and its precursors matched previously reported data in the literature.[10]

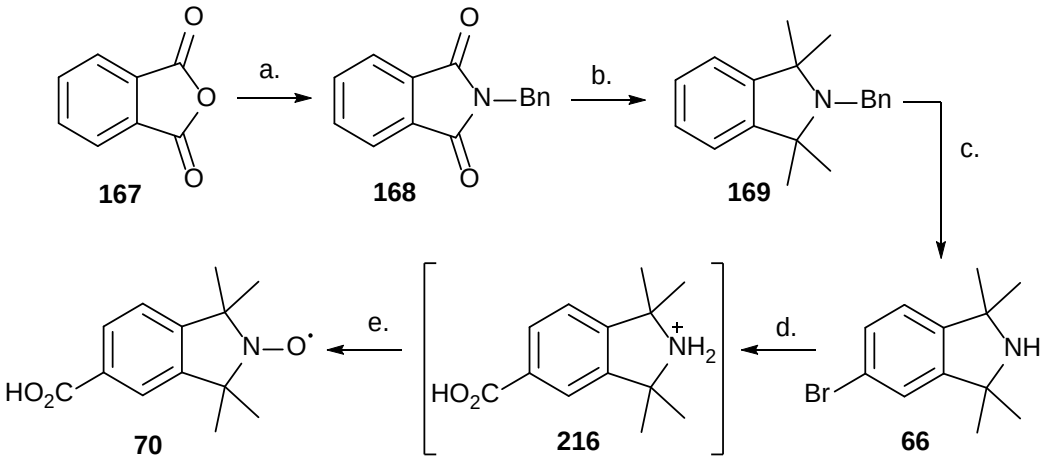
3.3.3 Approaches to the Synthesis of 5-Carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) 70

Though the applications of stable nitroxide compounds in various fields have been explored primarily with the piperidine and pyrrolidine type nitroxides, 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **70** (CTMIO), an isoindoline-based nitroxide, has displayed several promising biological properties in recent years.[13,14] Since its first reported synthesis 20 years ago, CTMIO possesses low cellular toxicity as well as high potential EPR sensitivity.[15] Unlike other isoindoline-based nitroxides which have limited biological application, the presence of the water solubilizing carboxyl group on CTMIO has allowed for its use as a potent antioxidant in a number of biological models and systems. In the autosomal recessive, genetic childhood disorder, *Ataxia telangiectasia* (AT), CTMIO significantly reduces oxidative stress in AT cells to a greater extent than known antioxidants such as vitamin E or Trolox.[16] CTMIO also acts as an almost-stoichiometric equivalent scavenger of protein radicals, thus reducing harmful cellular oxidation reactions and their associated damages.[17,18]

For synthetic purposes, CTMIO serves as an essential precursor to other isoindoline derivatives such as sulfonates and other carboxylic acid derivatives. However, CTMIO applications have been somewhat limited as it is not commercially available and its synthesis is laborious. Prior to this work, only one synthetic route to CTMIO (**Scheme 3.4**) had been established - a five step synthesis with an overall yield of 15%.[13] The key step involves the double lithiation of bromoamine precursor **66** followed by subsequent quenching of the dianion with carbon dioxide, to produce an *in situ* zwitterion. This zwitterion is difficult to handle and could not be easily isolated due to the presence of excess inorganic salts. Overall the highest yield achieved by a member of our research group at QUT over the two steps (d. and e.) was 62%. However, this yield was challenging to reproduce.

In an attempt to improve the yield and provide a practical approach for the synthesis of CTMIO, three alternative synthetic routes were explored as part of this project. The first two routes used the bromoamine precursor **66** to access CTMIO **70** via either the cyano derivative **71** or its benzaldehyde counterpart **73**. The third route

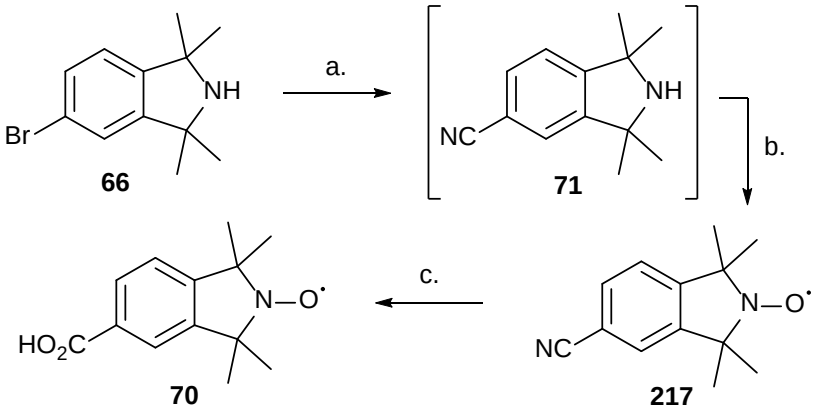
employed commercially available 4-methylphthalic anhydride **209** as the starting material, wherein the methylarene substituent could be subsequently oxidized to the desired carboxylic acid. The results are discussed below.



Scheme 3.4. Published synthetic route to CTMIO **70**. Reagents and conditions: a. $BnNH_2$, AcOH, reflux, 1 h, 98%; b. MeMgI, PhMe, reflux, 4 h, 27%; c. Br_2, $AlCl_3$, DCM, 0 ºC, 1 h, 91%; d. *n*-BuLi, THF, CO_2, -78 ºC, 10 min; e. H_2O/MeOH, $NaHCO_3$, $Na_2WO_4.2H_2O$, H_2O_2, RT, 48 h, 62%.

3.3.3.1 Synthesis of CTMIO 70 from 5-Cyano-1,1,3,3-tetramethylisoindoline 71

The first route to CTMIO **70** via the aminonitrile **71** is outlined in **Scheme 3.5** below. Using the copper (I)-catalyzed cyanation of arylbromides reported by Schareina *et al.*,[19] bromoamine **66** was treated with $K_4[Fe(CN)_6]$ in the presence of catalytic CuI and *N*-butylimidazole at elevated temperature to afford the aminonitrile derivative **71**. The FTIR spectrum of **71** showed a distinct sharp and strong C≡N vibration stretch at 2226 cm^{-1} and a weak N-H stretch at 3321 cm^{-1}. In comparison to the bromoamine precursor **66**, the extra peak in the unsaturated carbon region (111 ppm) in the ^{13}C NMR spectrum of aminonitrile **71** further supported the formation of a cyano functional group. Compound **71** could not be obtained in high purity (following analysis by HPLC) due to the presence of what was presumed to be a copper-*N*-butylimidazole complex that could not be completely removed by conventional purification techniques. However, the presence of the copper-*N*-butylimidazole complex did not affect the subsequent synthetic transformation. Other characterization data obtained for **71** included ^1H NMR and HRMS spectra.



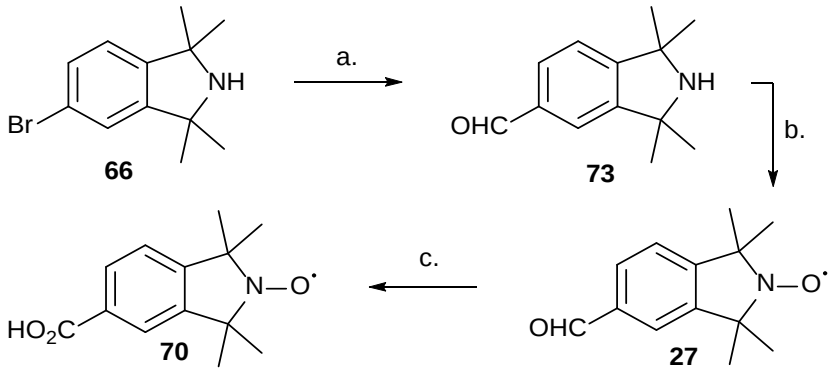
Scheme 3.5. Synthesis of CTMIO **70** from aminonitrile **71**. Reagents and conditions: a. $K_4[Fe(CN)_6].3H_2O$, CuI, *N*-butylimidazole, *o*-xylene, 180 ºC, 4 d, sealed vessel; b. *m*CPBA, DCM, 0 ºC 1 h to room temp., 3 h, 78%; c. NaOH, H_2O, EtOH, reflux, 16 h, 95%.

A mild peroxyl oxidation of the crude aminonitrile **71** with *m*CPBA afforded the corresponding cyano nitroxide **217** in 78% over two steps from **66**. The transformation was confirmed by the presence of a strong C≡N stretch and the loss of the weak N-H absorption at 2227 and 3321 cm^{-1} respectively in the FTIR spectrum of **217**. The $[M + 2H]^+$ ion for compound **217** was also observed at m/z 217 in the HRMS spectrum. Compound **217** was shown to be >95% pure by HPLC analysis. Due to significant paramagnetic broadening effect of the nitroxide moiety, no NMR spectroscopy data was obtained for compound **217**. Basic hydrolysis of compound **217** then gave the desired CTMIO **70**. The overall yield over three steps was 74%. The melting point determined for compound **70** matched that reported in the literature.[13]

3.3.3.2 Synthesis of CTMIO 70 from Formyl TMI 73

The second route to CTMIO **70** explored was through the formyl derivative **73** (**Scheme 3.6**). Firstly, bromoamine **66** was quantitatively converted to the formyl derivative **73** by quenching the resulting doubly lithiated anion (formed upon treatment of **66** with *n*-butyllithium) with DMF.[13] The formyl nitroxide **27**, obtained by *m*CPBA oxidation of **73**, was subsequently oxidized to the desired CTMIO **70** after treatment with a $AgNO_3$/NaOH solution over 5 h. This route gave an overall

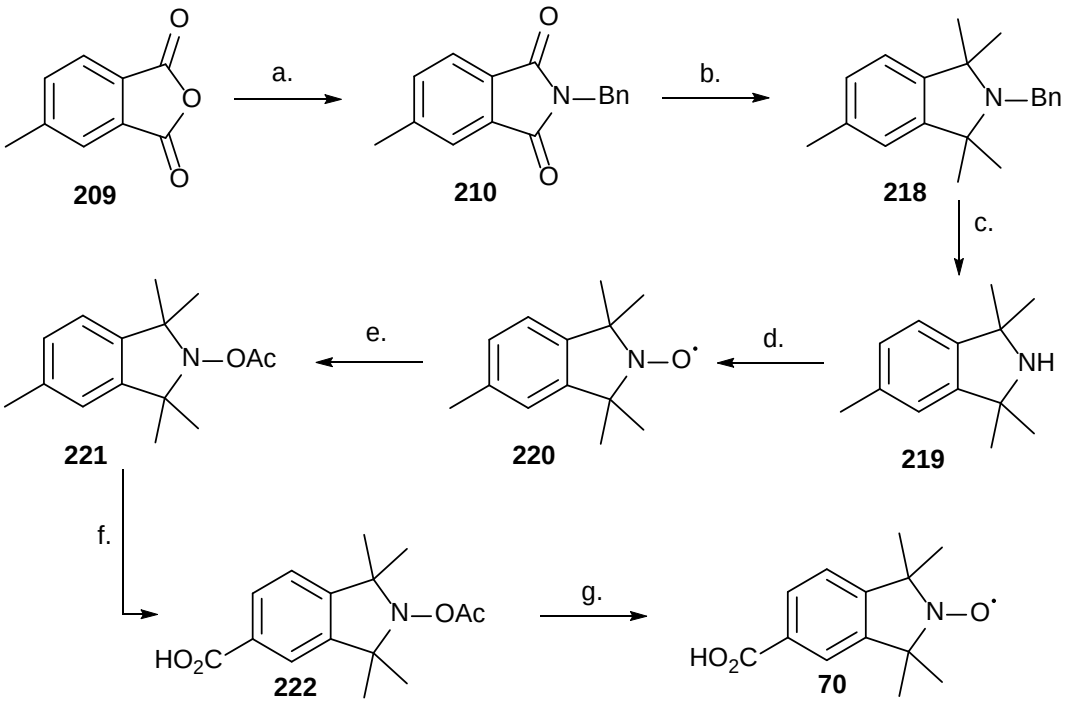
yield of 66% for CTMIO **70** from the compound **66** precursor. All characterization data obtained for compound **70** and its precursors matched that reported in the literature.[13]



Scheme 3.6. Synthesis of CTMIO **70** from formyl TMI **73**. Reagents and conditions: a. *n*-BuLi, THF, DMF, -78 ºC, 1 h to RT, 3 h, 100%; b. *m*CPBA, DCM, 0 ºC, 1 h, then RT, 3 h, 92%; c. $AgNO_3$, NaOH, H_2O, RT, 6 h, 72%.

3.3.3.3 Synthesis of CTMIO 70 from 4-Methylphthalic Anhydride 209

The final alternative route to CTMIO **70** was explored using 4-methylphthalic anhydride **209** as the starting material (**Scheme 3.7**). *N*-Benzyl-4-methylphthalimide **210** was obtained in excellent yield (98%) after an acetic acid solution of 4-methylphthalic anhydride **209** and benzylamine was refluxed for 1 h.[10] Exhaustive methylation of phthalimide **210** with a six fold excess of freshly prepared methylmagnesium iodide furnished compound **218** in a modest yield of 34%. The two singlets at 1.30 and 1.31 ppm, each integrating for six protons, in the ^{1}H NMR spectrum of **218** confirmed the successful transformation. The aryl methyl group and benzylic CH_2 protons were also evident in the ^{1}H NMR spectrum at 2.39 and 4 ppm respectively. The nitroxide **220** was obtained following quantitative debenzylation of compound **218** with hydrogen gas and catalytic palladium on activated charcoal, and subsequent *m*CPBA oxidation of the resulting secondary amine **219**.



Scheme 3.7. Synthesis of CTMIO **70** from 4-methylphthalic anhydride **209**. Reagents and conditions: a. $BnNH_2$, AcOH, reflux, 1 h, 98%; b. MeMgI, PhMe, reflux, 4 h, 34%; c. H_2 (50 psi), Pd/C, AcOH, RT, 4 h, 96%; d. *m*CPBA, DCM, RT, 3 h, 95%; e. AcCl, Et_3N, 0 °C, 30 min. to RT, 1 h, 91%; f. $KMnO_4$,*t*-BuOH, $MgSO_4$, 70 °C, 1 d, 65%; g. NaOH, overnight, 97%.

As mentioned in a preceeding section, nitroxides do not survive permangante oxidation.[10] It was therefore necessary to protect the nitroxide moiety of compound **220** with an acetyl protecting group. Hence, Pd/C-catalyzed hydrogenolysis of the nitroxide **220** afforded the corresponding hydroxyamine, which was acetylated *in situ* with acetyl chloride in the presence of triethylamine to furnish compound **221** (95%). The presence of the *N*-acetoxy protecting group was verified in the NMR and FTIR spectra of **221**. The protons of the acetyl group were observed as a singlet at 2.2 ppm in the ^{1}H NMR spectrum and a strong C=O stretch was also observed at 1758 cm^{-1} in the FTIR spectrum.

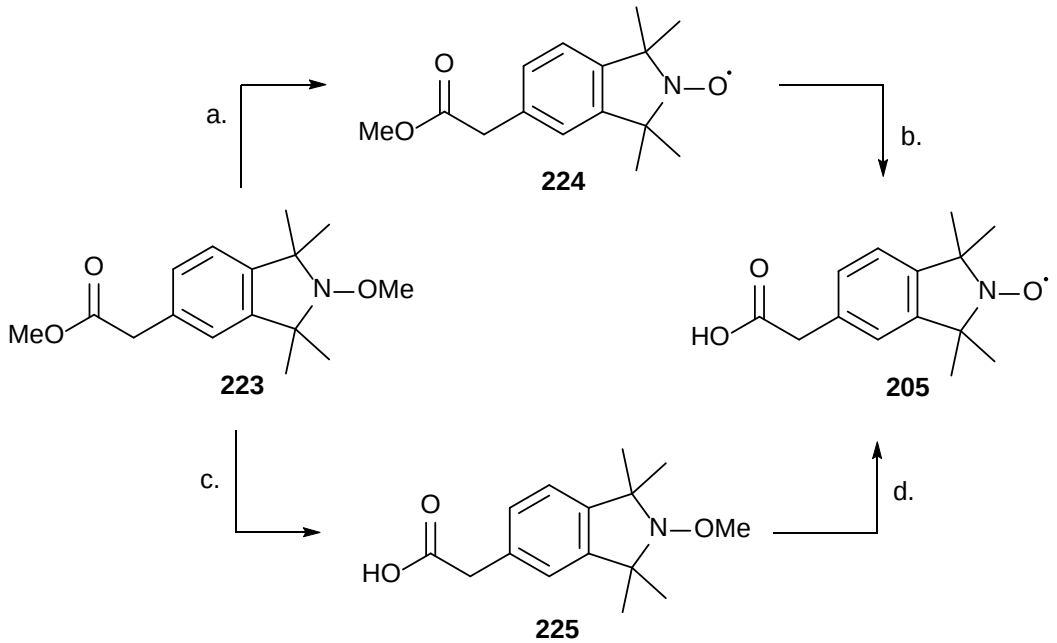
A regio-selective potassium permanganate side chain oxidation of the aryl methyl group of **221** afforded the corresponding carboxylic acid **222** in moderate yield (65%). In addition to the *N*-acetoxy C=O stretch at 1771 cm^{-1}, a strong C=O stretch

at 1681 cm^{-1} and a medium broad O-H stretch (3400 to 2800 cm^{-1}) were observed in the FTIR spectrum of **222**. These evidently supported the presence of a carboxylic acid functional group. The structure of the *N*-acetoxy protected carboxylic acid **222** was further confirmed by HRMS, ^{1}H and ^{13}C NMR spectroscopies. The carboxylic acid **222** was readily deprotected to give CTMIO **70** after stirring overnight in dilute sodium hydroxide solution. The overall yield of CTMIO from this route was 17.4%.

Athough each of the three new alternative synthetic approaches to CTMIO **70** gave comparative overall yields (16-18%), the nitrile route gave the best overall yield (18%). However, the fastest route to CTMIO **70** is through the formyl derivative **73**.

3.3.4 Synthesis of 5-Carboxymethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 205

The carboxylic acid **205** was synthesized from the methyl ester intermediate **223** as shown in **Scheme 3.8** below. The synthesis of methyl ester **223** is discussed in **Chapter 4**.



Scheme 3.8. Synthesis of 5-carboxymethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **205**. Reagents and conditions: a. *m*CPBA, DCM, 3 h, 89%; b. MeOH/NaOH, 60 ^{o}C, 2 h, 94%; c. MeOH/NaOH, 60 ^{o}C, 96%; d. *m*CPBA, DCM, 3 h.

First, methyl ester **223** was readily converted to the nitroxide derivative **224** under mild *m*CPBA oxidation conditions. Compound **224** was obtained as a yellow solid in 89% yield after purification of the crude product by silica gel column chromatography. The identity of **224** was confirmed by HRMS and FTIR analysis. A strong C=O stretching frequency was observed at 1737 cm^{-1} in the FTIR spectrum. In addition, the masses observed at m/z 263 and 264 in the HRMS spectrum of **224** were consistent with the mass of the expected $[M + H]^+$ and $[M + 2H]^+$ ions respectively. The purity of compound **224** was assessed by HPLC analysis to be >95% and its melting range was determined to be 100-101 ^oC.

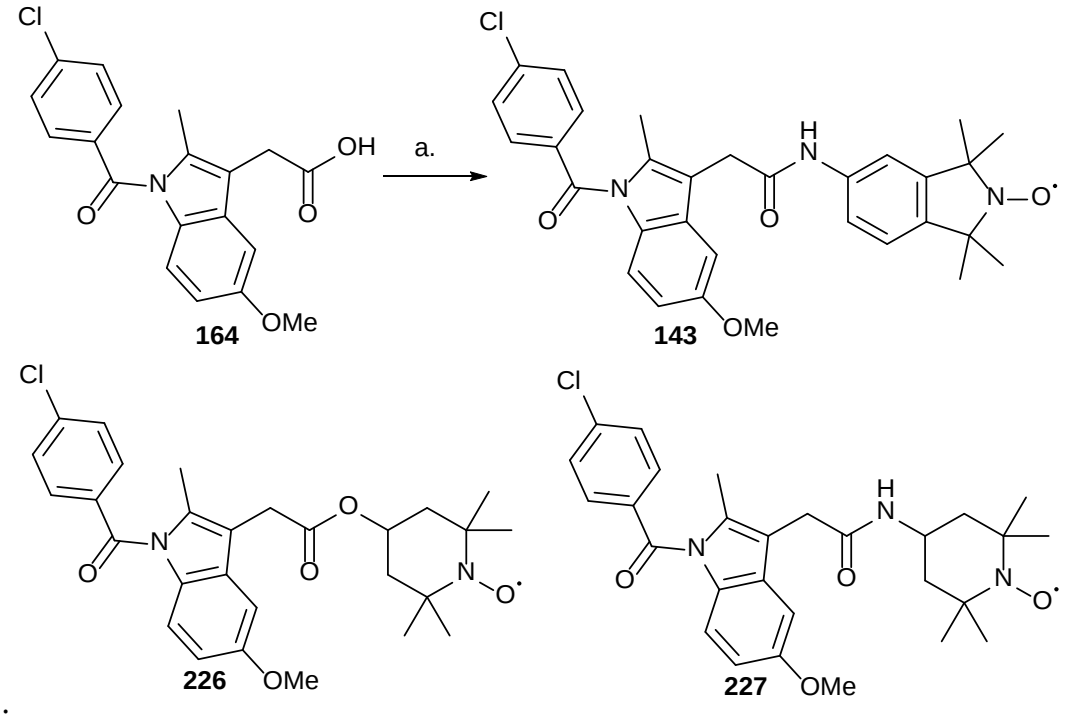
The carboxylic acid nitroxide derivative **205** was obtained almost quantitatively by basic hydrolysis of the methyl ester **224** with sodium hydroxide. In the FTIR spectrum of **205**, characteristic broad OH and strong C=O absorptions were observed at 3520 and 1650 cm^{-1} respectively. This evidently supported the presence of the carboxylic acid functional group. Also, the molecular ion plus proton $[M + H]^+$ was observed at m/z 249 in the HRMS spectrum of **205**.

Likewise, the carboxylic acid nitroxide **205** was also obtained by *m*CPBA oxidation of the corresponding methoxyamine **225**. In this case however, it was difficult to separate compound **205** from the *meta*-chorobenzoic acid by-product by conventional purification techniques (such as chromatography and recrystallization). The next sections explore the synthesis of the novel nitroxide-NSAID conjugates by carbodiimide chemistry.

3.3.5 Synthesis of Nitroxide-Indomethacin Conjugates

Like aspirin, indomethacin is a conventional NSAID that inhibits both COX-1 and COX-2 enzymes. Such nonselectivity towards the COX enzyme is linked to the observed gastrointestinal toxicity (GI ulceration and bleeding) that usually arises after prolong use of indomethacin even at relatively low doses. Studies have shown that slight structural changes in indomethacin (or aspirin) alter its inhibitory action.[20-23] For instance, extending the carboxylic acid side chain or replacing the 4-chlorobenzoyl moiety with a 4-bromobenzyl group result in high selectivity towards the COX-2 enzyme.[20] In contrast to the parent indomethacin, amides and ester

derivatives display superior selectively to the COX-2 enzyme with improved GI safety profiles even at relatively high doses above their therapeutic efficacy.[23,24] Herein, similar structural modifications were carried out on indomethacin to give novel nitroxide-indomethacin cleavable conjugates. As outlined in **Scheme 3.9** below, the preparation of nitroxide-indomethacin conjugate **143** began by reacting 5-amino TMIO **108** with indomethacin **164** under EDC coupling conditions similar to those used in the preceding sections.



Scheme 3.9. Synthesis of nitroxide-indomethacin conjugates (**143, 226** and **227**). Reagents and conditions: a. nitroxide (compound **76**, **102** or **108**), EDC, DMAP, DCM, RT, 1 d, 83-91%.

The amide-linked nitroxide-indomethacin conjugate **143** was obtained as a bright yellow crystalline solid in 90% yield following purification by silica gel column chromatography and then recrystallization. TEMPOL **102** and 4-amino-TEMPO **76** were also subjected to similar coupling conditions to give the nitroxide indomethacin conjugates **226** and **227** respectively. Characterization data obtained to support the successful synthesis of each nitroxide indomethacin conjugate included HRMS, FTIR and ^{1}H and ^{13}C NMR spectra. The FTIR spectrum of compound **143** for

instance showed a weak N-H stretch at 3299 cm^{-1} along with two characteristic amide C=O stretching frequencies at 1677 and 1599 cm^{-1}.

As mentioned in the preceding section, characterization of nitroxide containing compounds by NMR spectroscopy is quite challenging. This is due to the paramagnetic line broadening effect commonly observed in the NMR spectrum. The broadening effect arises from dipolar interactions between the unpaired electron of the nitroxide moiety and nearby nuclei (protons and carbons).[25,26] Unlike dipole-dipole interactions between protons (which are limited to short distances up to 5.5 Å), the interactions between the unpaired electron of the nitroxide moiety and nearby nuclei extends over long distances (up to 25 Å).[27] Thus, such proportionate distance-dependent line broadening means that the paramagnetic effect is more pronounced for nuclei in close proximity to the nitroxide spin. However, using lower concentration of nitroxide in the samples can in some instances, reduce the line broadening effect.

Hence, for compounds such as the nitroxide-indomethacin conjugates **143, 226** and **227**, it was possible to obtain good structural information for $^{1}H/^{13}C$ nuclei of sufficient distance from the nitroxide radical by NMR spectroscopy. **Figure 3.4** below shows the ^{1}H NMR spectrum obtained from a dilute sample of conjugate **226**. The singlets at 2.44, 3.7 and 3.86 ppm are characteristic of the methyl, methylene and methoxy protons of the indomethacin side chain respectively. The two doublets at 6.7 and 6.9 ppm and the adjacent singlet at 6.99 ppm are consistent with the three aromatic protons of the indole ring with the expected coupling patterns. The two distinct protons of the *para*-chloro substituted aromatic ring are shown as doublets at 7.5 and 7.7 ppm. Almost all carbon atoms of the indomethacin structure were successfully assigned in the ^{13}C NMR spectrum of **226**. A similar trend was observed for the nitroxide-indomethacin conjugate **143**. Despite a more pronounced line broadening effect in the ^{1}H NMR spectrum of the amide conjugate **227**, the diagnostic proton signals for the indomethacin side chain were also assigned.

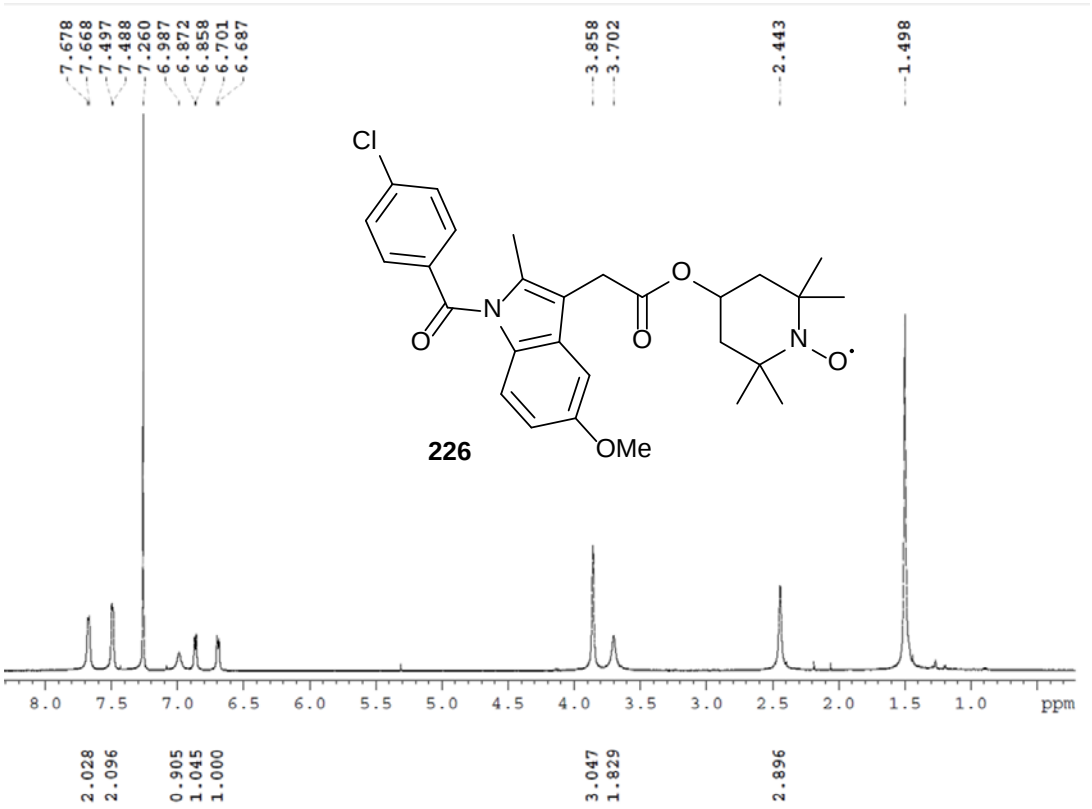
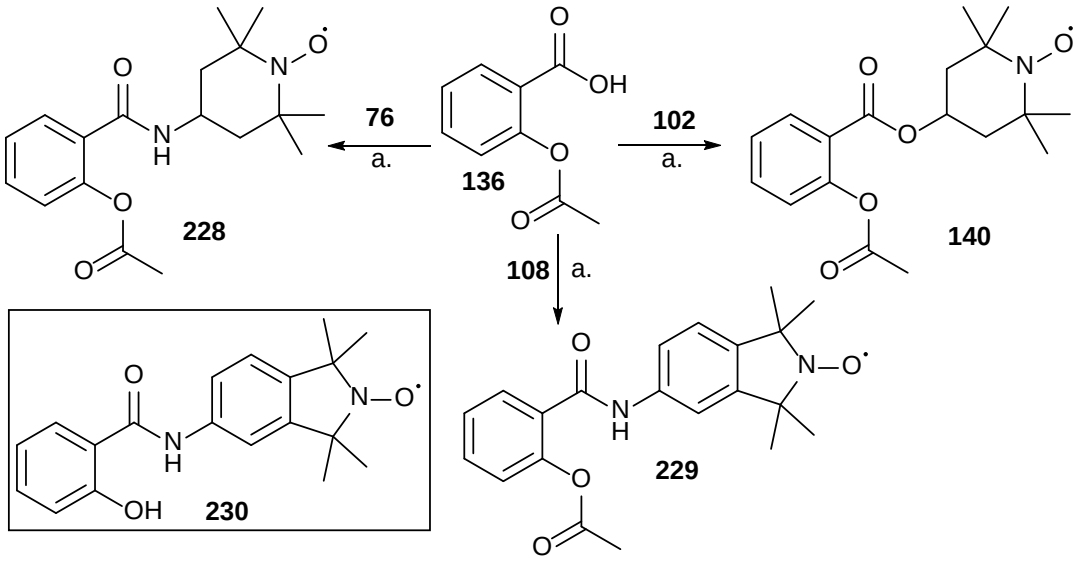


Figure 3.4. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of nitroxide-indomethacin conjugate **226**.

3.3.6 Synthesis of Nitroxide-Salicylate Conjugates

The first series of nitroxide-aspirin cleavable conjugates (**140, 228** and **229**) was synthesized by attaching the various nitroxide compounds directly to the carboxylic acid group of aspirin **136** through amide and ester linkages. As outlined in **Scheme 3.10** below, 4-amino-TEMPO **76** and 5-amino-TMIO **108** were used to synthesize the amide nitroxide-aspirin conjugates **228** and **229** respectively. TEMPOL **102** was also succesfully linked to aspirin **136** by esterification to give the ester conjugate **140**. Each of these transformations was achieved by similar carbodiimide coupling conditions. Briefly, almost equimolar amounts of nitroxide, aspirin and EDC, and a catalytic amount of DMAP were reacted at room temperature for 1 day. Although the formation of compound **140** proceeded smoothly, the synthesis of **229** under similar coupling conditions was accompanied by the formation of, surprisingly, the *ortho*-deacetylated amide **230** (32%). This was verified by the presence of a strong broad OH stretch at 3400 cm^{-1} and the loss of the characteristic acetyl group C=O stretch

around 1640 cm^{-1} in the FTIR spectrum of **230**. Further evidence to support the formation of **230** was obtained from the presence of the $[M + H]^+$ ion at m/z 325 in the HRMS spectrum. TLC analysis of the reaction mixture indicated that the formation of both the desired product **229** and the *ortho*-phenolic amide side-product **230** began immediately after the start of the reaction. The mixture was separated by silica gel chromatography.



Scheme 3.10. Synthesis of the first series of aspirin nitroxide conjugates (**140, 228** and **229**). Reagents and conditions: a. EDC, DMAP, DCM, RT, 1 d, 53-90%.

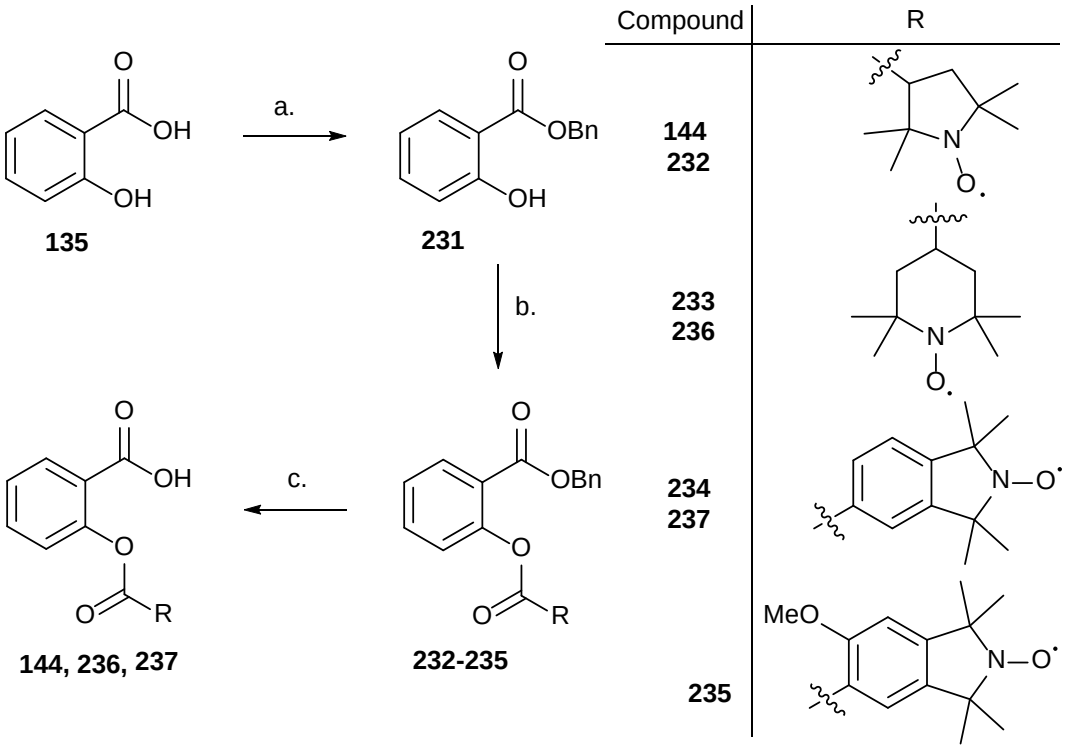
The *ortho*-phenolic amide side-product was likely formed from hydrolysis of the *ortho*-acetate group of either aspirin **136**, or the desired product **229**, or the *o*-acylisourea and anhydride intermediates (see **Scheme 3.1**). Notably, in the absence of the basic DMAP catalyst, no side reaction was observed. In this case, however, less than 8% of starting material was converted to the desired product after four or more days. Similar *o*-deacetylation was observed in the synthesis of the amide conjugate **228**. This indicated that the hydrolysis is likely facilitated by the presence of DMAP as well as the amino nitroxide precursors (**76** and **108**).

The cleavable conjugates (**140**, **228** and **229**) were obtained in good to high yields (53-90%) after purification by column chromatography. The products were characterized by FTIR and HRMS analysis, and their purity assessed by HPLC (>95%). The medium N-H stretch (at 3314 cm^{-1}) and the extra strong C=O

absorption (at 1546 cm^{-1}) in the FTIR spectrum of **228** supported the presence of an amide bond. Also, no characteristic O-H absorption, typical for carboxylic acid (a strong, broad band above 3000 cm^{-1}), was present in the FTIR spectrum. This further indicated the successful transformation of aspirin to the amide derivative **228**. Further evidence to confirm the identity of **228** was obtained by HRMS with the presence of the $[M + H]^+$ ion at m/z 334.

For the next series of nitroxide-aspirin conjugates, the aim was to link various carboxylic acid containing nitroxides to salicylic acid **135** via ester bonds. The carboxylic acid bearing nitroxides used include 4-carboxy TEMPO **8**, CTMIO **70**, 6-methoxy-CTMIO **175** and carboxy PROXYL **204**. However, as both reactants (salicylic acid **135** and the nitroxide) have carboxylic acid functionality, they can each react under carbodiimide coupling conditions to independently form the active *o*-acylisourea intermediate (**v**). This may lead to the formation of unwanted side products (such as the stable *N*-acylurea (**x**) or a salicylate dimer) and decrease the yield of the desired product. To overcome such potential drawbacks, salicylic acid **135** was first converted to the benzyl salicylate **231** (**Scheme 3.11**). This was achieved by reacting equimolar amounts of salicylic acid **135** and benzyl bromide in the presence of sodium bicarbonate. The benzyl salicylate **231** was obtained in 94% yield as a clear oil. All characterization data obtained for compound **231** matched that in the literature.[28]

The benzyl salicylate **231** was then reacted with various carboxylic acid nitroxides under EDC/DMAP coupling conditions to give compounds **232-235** in high yields (85-90%). The identity of each product was confirmed by ^{1}H NMR and FTIR spectroscopy, and HRMS. In the ^{1}H NMR spectrum of compound **234** for instance, the strong, broad singlet at 5.25 ppm was assigned to the distinctive CH_2 protons of the benzylic ester protecting group. Almost all salicylate and benzyl aromatic protons were also observed for each benzyl salicylate-nitroxide (**232-235**) in the respective ^{1}H NMR spectrum. The presence of the two characteristic C=O absorptions (at 1748 and 1718 cm^{-1}) and the disappearance of an O-H stretch (from either reactants, **70** and **231**) in the FTIR spectrum indicated successful transformation.

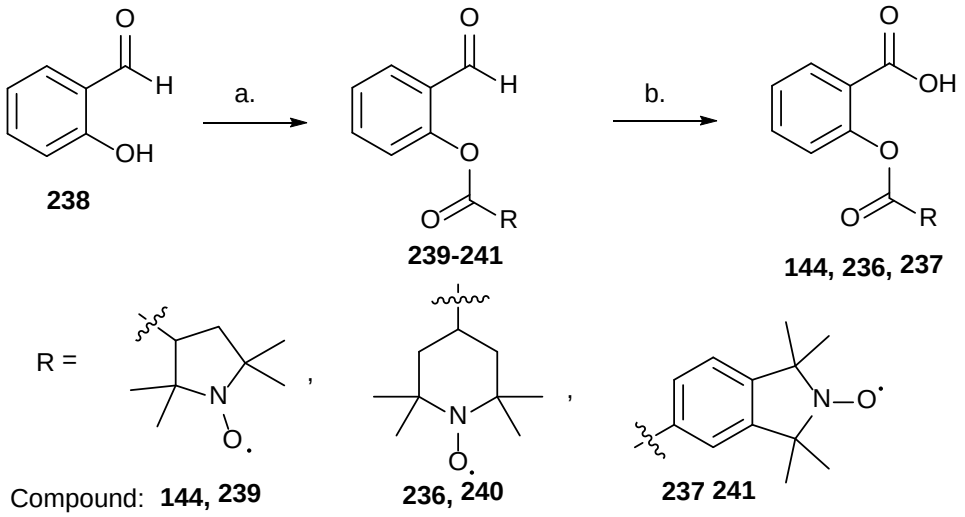


Scheme 3.11. Synthesis of the second series of salicylate nitroxide conjugates via benzyl salicylate **231**. Reagents and conditions. a. BnBr, $NaHCO_3$, DMF, overnight, 50 ^oC, 94%; b. Appropriate carboxylic acid nitroxide (compound **8**, **70**, **175** or **204**), EDC, DMAP, DCM, RT, 1 d, 85-90%; c. H_2, Pd/C, MeOH, overnight, 41-49%.

The next step was to debenzylate the *o*-nitroxide salicylates (**232-234**) to the corresponding acid derivatives (**144, 236** and **237**). This was achieved using palladium catalyzed hydrogenation conditions. Although complete conversion was achieved and no side reactions were observed, the isolated yields for each of the debenzylated products (**144, 236** and **237**) was, surprisingly, quite low (41-49%). Each target nitroxide-salicylate conjugate (**144, 236** and **237**) was characterized by ^1H NMR and FTIR spectroscopy and HRMS. Although the carboxylic acid functional group was not observed in the ^1H NMR spectra of the target conjugates (**144**, **236** and **237**), signals for the four distinct salicylate aromatic ring protons were present. The disappearance of the benzyl group protons of the benzyl salicylate precursors (**232-234**) at 5.25 ppm and around 7.26 ppm further supported the successful transformations. The medium broad absorption observed in the FTIR

spectrum of **236** (3550 to 2500 cm^{-1}) is a characteristic O-H stretch. This, along with the two strong C=O stretches (at 1755 and 1716 cm^{-1}), supported the presence of a carboxylic acid and an ester. The poor yields were initially attributed to the products being lost during the filtration process or trapped within the Pd/C residue. However, the yields could not be improved even after several extractions of the Pd/C solid residue with dichloromethane and ethyl acetate. The presence of the second ester bond linking each nitroxide in the intermediates to the benzyl salicylate, means that it is unlikely that these compounds be selectively debenzylated by acid or base hydrolysis.

Therefore, an alternative, but convenient, approach was pursued for the synthesis of the salicylate conjugates (**144**, **236** and **237**) starting from salicylaldehyde **238**. As shown in **Scheme 3.12** below, EDC coupling of carboxylic acid nitroxides (compound **8**, **70** or **204**) with salicylaldehyde **238** afforded the nitroxide *o*-formyl phenyl ester derivatives (**239-241**) in high yields (83-91%).



Scheme 3.12. Synthesis of the second series of salicylate nitroxide conjugates via salicylaldehyde **238**. Reagents and conditions: a. Appropriate carboxylic acid nitroxide (compound **8**, **70** or **204**), EDC, DMAP, DCM, RT, overnight, 83-91%; b. $NaClO_2$, NaH_2PO_4, H_2O_2, MeCN, 0 ^oC-RT, 80-88%.

The structure and purity of the nitroxide *o*-formyl phenyl ester derivatives (**239-241**) were verified by HRMS, FTIR and ^1H NMR spectroscopy and HPLC. The singlet at 10.21 ppm in the ^1H NMR spectrum of compound **241** was assigned to the aldehyde

proton. The spectrum also confirmed the presence of the four aromatic protons, with their expected coupling patterns. The C=O absorption of both the aldehyde and the ester functional groups were shown as strong bands at 1749 and 1700 cm^{-1} respectively in the FTIR spectrum of **241**.

Although a variety of methods are used to oxidize aldehydes to carboxylic acids, the Pinnick oxidation method was employed to convert the *o*-formyl phenyl esters (**239-241**) to the corresponding acid derivatives (**144, 236** and **237**). The Pinnick oxidation reaction uses sodium chlorite ($NaClO_2$) under mild acid conditions as the source of the active oxidizing agent.[29-31] The acid, most commonly sodium dihydrogen phosphate (NaH_2PO_4), acts as a buffer reacting with $NaClO_2$ to generate the active oxidizing agent, chlorous acid.

Thus, each *o*-formyl nitroxide (**239-241**) was oxidized to its salicylate derivative (**144, 236** and **237**) by allowing it to react with $NaClO_2$ in the presence of hydrogen peroxide and dihydrogen phosphate. The hydrogen peroxide is employed to quench the hypochlorite (HOCl) by-product that is usually generated in this reaction condition. Hypochlorite is a stronger oxidant and if not trapped, it can readily oxidize the required $NaClO_2$ oxidant and other functional substrates.[32] The target salicylate conjugates (**144, 236** and **237**) were obtained in high yields (80-88%) after purification by silica gel column chromatography and recrystallization. Alternatively, each *o*-formyl ester (**239-241**) was readily oxidized to the carboxylic acid derivative under mild potassium permanganate oxidation conditions in 1 h. Similar high yields were again obtained. The purity and characterization data obtained for the carboxylic acid conjugates (**144, 236** and **237**) obtained via this route (Pinnick oxidation) were identical to those obtained from the benzyl protected intermediates (**232-234**). The lowest overall yield over the two step formyl route was 66% while that of the three step benzyl benzoate ester route was 16%.

3.3.7 Synthesis of Nitroxide-5-Aminosalicylic Acid (5-ASA) Conjugates

In contrast to other conventional NSAIDs, such as aspirin, that cause GI toxicity, 5-aminosalicylic acid exerts its therapeutic action on inflammatory diseases of the GI

track. The four 5-aminosalicylic acid derivatives currently on the drug market include mesalamine **242**, sulfasalazine **243**, balsalazide **244** and olsalazine **245** (**Figure 3.5**).[33] These derivatives are the mainstay of treatment for inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC).[34-36] Ulcerative colitis is characterized by persistent mucosal inflammation of the colon that leads to abdominal pain, anemia, bleeding, diarrhea, and weight loss. Despite the GI tolerance of 5-ASA derivatives, combining them with antioxidant agents such as stable nitroxides may enhance their anti-inflammatory efficacy by reducing oxidative stress. Thus, the following sections will examine the synthesis of amide and amine nitroxide conjugates of 5-ASA.

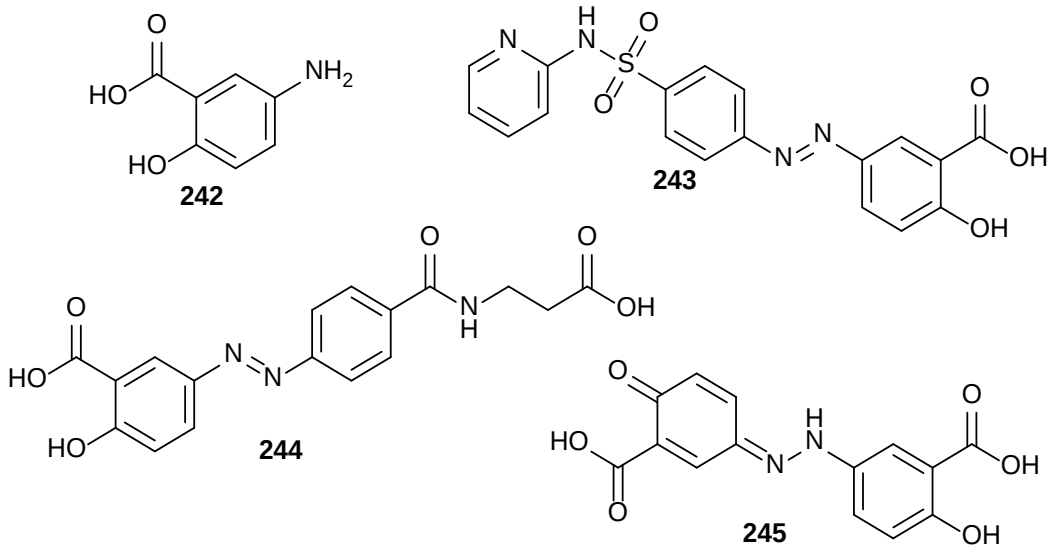
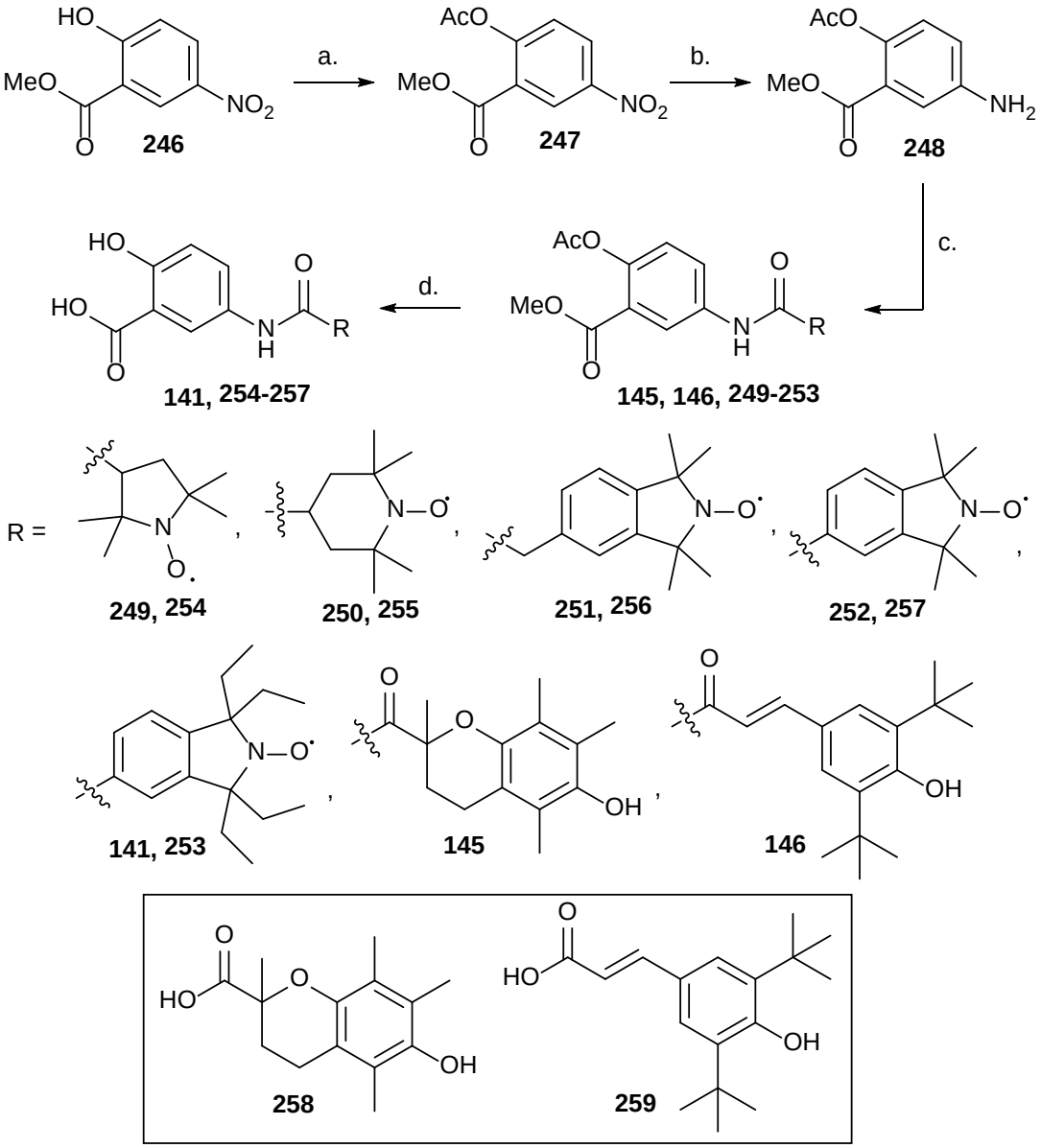


Figure 3.5. Common 5-aminosalicylic acid derivatives: mesalamine **242** (5-ASA), sulfasalazine **243**, balsalazide **244** and olsalazine **245**.

3.3.7.1 Synthesis of Amide-linked Nitroxide-5-ASA Conjugates

A number of nitroxides were incorporated into the 5-ASA framework via carbodiimide-facilitated amide bond formation starting from methyl 2-hydroxy-5-nitrobenzoate **246** (**Scheme 3.13**). The phenol group of methyl 2-hydroxy-5-nitrobenzoate **246** was first protected with an acetyl group by reacting it with acetyl chloride in the presence of triethylamine to furnish the nitro-diester **247**. The appearance of the characteristic methyl proton singlet of the acetyl protection group at 2.4 ppm in the ^{1}H NMR spectrum of compound **247** supported the formation of the

acetyl protected methyl benzoate. Compound **247** was then reduced to the amine derivative **248** under Pd/C hydrogenolysis. All characterization data (NMR and FTIR spectroscopy and melting point) obtained for compound **248** matched that previously reported in the literature.[37]



Scheme 3.13. Synthesis of amide-linked nitroxide-5-ASA conjugates. Reagents and conditions: a. AcCl, TEA, 0 ºC, 30 min. to RT, 1 h, 91%; b. H_2, Pd/C, EtOAc, 50 psi, 5 h, 90%; c. Appropriate carboxylic acid (**8**, **70, 204-206, 258** or **259**), EDC, DMAP, DCM, RT, 1 d, 83-91%; d. 1 M NaOH/MeOH, overnight, 83-91%.

The amino ester **248** was then coupled to various carboxylic acid nitroxides under EDC coupling conditions to give the amide derivatives (**249-253**). To compare the therapeutic efficacy of the novel nitroxides conjugates to known pharmacophores, the 5-ASA conjugates of known antioxidants trolox **258** and cinnamic acid **259** were also synthesized (**145** and **146**). The identity and purity of each product (**145, 146, 249-253**) was confirmed by NMR and FTIR spectroscopy, HRMS, and HPLC analysis. A mild basic hydrolysis of the amide-diesters (**249-253**) afforded the desired amide salicylates (**141, 254-257**) in high yields (83-91%).

3.3.7.2 Synthesis of Amine-Linked Nitroxide-5-ASA Conjugate

In addition to the amide conjugates, the amino-linked conjugate **142** was the ultimate target of this chapter (**Figure 3.6**). Compound **142** is an intriguing target that is similar in structure to 2-hydroxy-5-[2-(4-trifluoromethylphenyl)-ethylaminobenzoic acid **260** (AAD-2004). AAD-2004, an amine derivative of 5-ASA (with ethylamino linker), is a potent antioxidant that displayed promising neuroprotective action on neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS).[38,39]

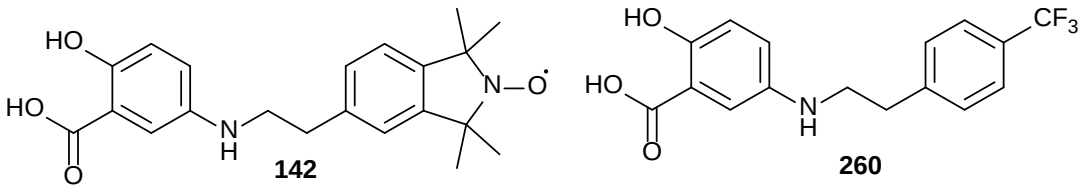
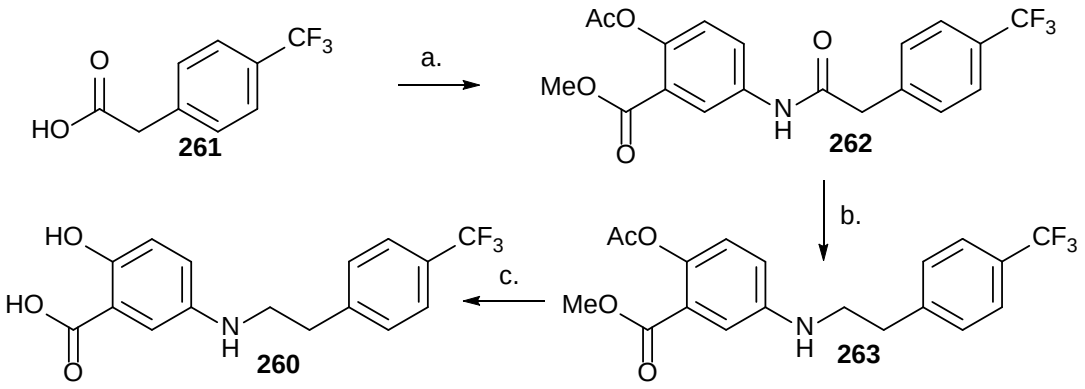


Figure 3.6. Chemical structures of AAD-2004 **260** and the target nitroxide derivative **142**.

With the amide derivative **251** in hand, the challenge was to selectively reduce the amide bond of conjugate **251** to the corresponding amine derivative without reducing the ester groups. Recently, Yoon and coworkers reported the synthesis of AAD-2004 **260** and a series of 5-(*N*-phenethylamino)salicylic acid derivatives in three steps from methyl 2-acetoxy-5-aminosalicylate ester **248** and the corresponding phenylacetic acid derivatives (**Scheme 3.14**).[40]

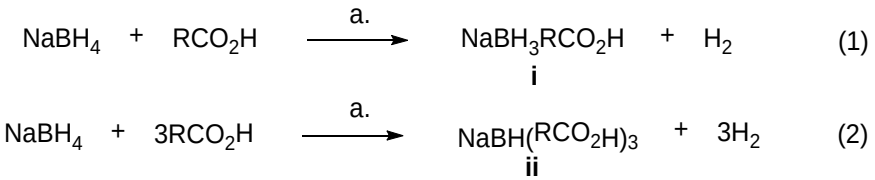
The key step in their approach was the selective reduction of the amide group of the intermediate **262** to the 5-(*N*-phenethylamino) derivative **263**. This was achieved in

good yield with sodium acyloxyborohydride as the reducing agent. Acyloxyborohydrides are versatile synthetic reagents known to selectively reduce amides to the corresponding amines.[40-42]



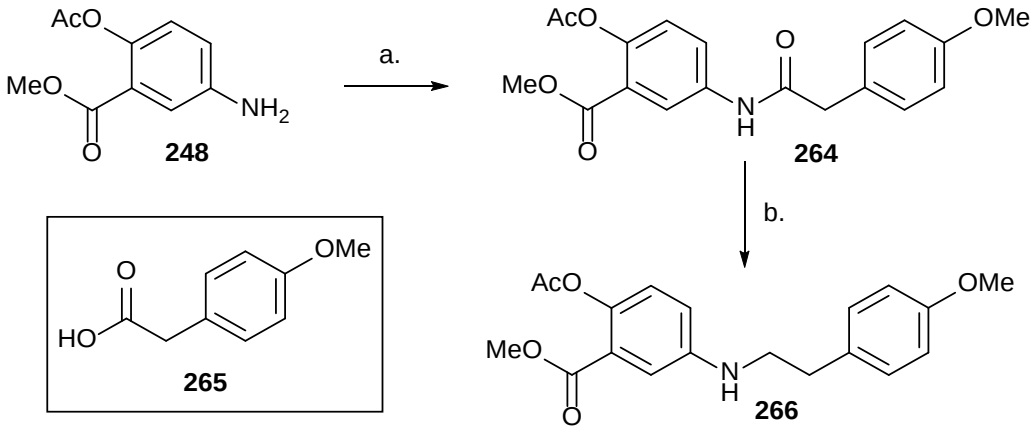
Scheme 3.14. Synthesis of AAD-2004 via selective amide reduction using the sodium acyloxyborohydride reagent. Reagents and conditions: a. Compound **248**, EDC, DMAP, DCM; b. $NaBH_4$, AcOH, dioxane, reflux, 64%; c. 6 N HCl, AcOH, reflux, 85%.

Sodium acyloxyborohydride is a modified-$NaBH_4$ reagent formed when $NaBH_4$ is reacted with a carboxylic acid (most commonly, acetic acid or trifluoroacetic acid) in aprotic solvents (**Scheme 3.15**).[42] For amide reduction, the active reducing agent is the more reactive acyloxyborohydride (**i**). Therefore, equimolar amounts of the acid and $NaBH_4$ are required for the optimum formation of acyloxyborohydride (**i**). Also, careful (slow) addition of the acid reagent to a suspension of $NaBH_4$ in the appropriate solvent reduces the formation of the the less reactive triacyloxyborohydride complex (**ii**).



Scheme 3.15. Sodium acyloxyborohydride formation. Typical reaction conditions: a. THF, -30 ^{o}C-RT.

To evaluate the selectivity of this reagent on the 5-ASA amide conjugates, a model reaction was first attempted with methyl 2-acetoxy-5-(2-(4-methoxyphenyl)acetamido)benzoate **264** (**Scheme 3.16**). The methyl 2-acetoxy-5-[2-(4-methoxyphenyl)acetylamino]benzoate ester **264** was readily synthesized by reacting 2-(4-methoxyphenyl)acetic acid **265** with methyl 2-acetoxy-5-aminobenzoate **248** under EDC coupling conditions. Compound **264** was then selectively reduced to the amine derivated **266** by allowing it to react for 25 min with a preformed solution of sodium acyloxyborohydride in refluxing dioxane. The selective reduction of the amide group was confirmed by the presence of the two triplets, each integrating for two protons, at 2.86 and 3.36 ppm in the ^{1}H NMR spectrum of **266** (**Figure 3.7**).



Scheme 3.16. Model reaction for the selective amide reduction with sodium acyloxyborohydride reagent. Reagents and conditions: a. Compound **265**, EDC, DMAP, DCM, RT, 1 d, 90%; b. $NaBH_4$, AcOH, dioxane, reflux, 25 min, 66%.

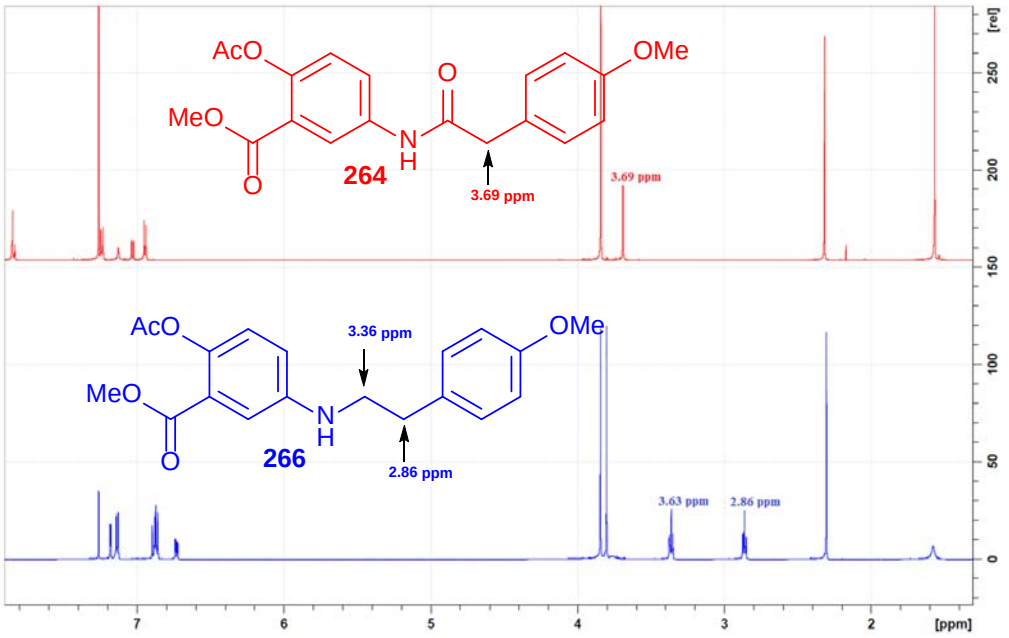
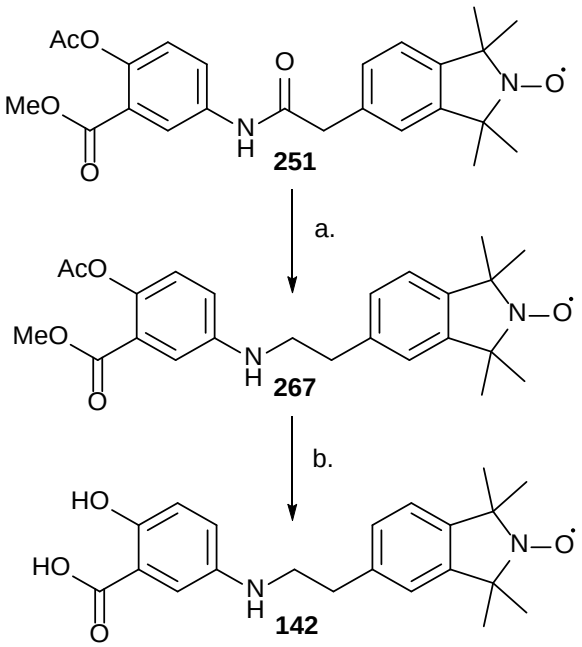


Figure 3.7. Comparison of ^{1}H NMR (600 MHz) spectra of amide precursor **264** and its 5-ethylamino derivative **266**.

The methyl protons of both esters (acetotoxy and methyl ester) were also observed in the ^{1}H NMR spectrum. Though compound **266** was obtained in 66% yield, the reduction of **264** was accompanied by the formation of less than 10% of a mixture of the reduced esters as well. This was verified by the ^{1}H NMR spectrum of the crude product mixture.

Having established the optimal conditions for the selective reduction of the 5-ASA amide, the next step was to apply these conditions to the synthesis of the nitroxide-5-ASA amino conjugate **142**. Accordingly, when methyl 2-acetoxy-5-[1,1,3,3-tetramethylisoindolin-2-yloxyl)acetylamino] benzoate ester **251** was subjected to similar sodium acyloxyborohydride reduction conditions, the 5-ethylamino derivative **267** was obtained in 68% yield following purification by silica gel column chromatography and recrystallization (**Scheme 3.17**).



Scheme 3.17. Synthesis of amine-linked nitroxide-5-ASA conjugate **142** using sodium acyloxyborohydride reduction conditions. Reagents and conditions: a. $NaBH_4$, AcOH, dioxane, reflux, 30 min, 68%; b. 0.5 M NaOH/MeOH, overnight, 72%.

Characterization data including FTIR, HRMS, and ^{1}H NMR spectra supported the successful synthesis of compound **267**. Only two distinct C=O stretching frequencies were observed (at 1746 and 1715 cm^{-1}) in the FTIR spectrum. This is consistent with the presence of the acetyl and methyl benzoate groups. A sharp medium N-H stretch of the ethylamino linker was also observed at 3371 cm^{-1}.

Despite the expected paramagnetic broadening effect of the nitroxide moiety, the methyl protons of both esters (acetoxy and methyl ester) were also observed in the ^{1}H NMR spectrum of **267** at 2.31 and 3.85 ppm respectively (**Figure 3.8**). When compared to the model compound **266** (**Figure 3.7**), the weak broad peak at 3.44 ppm likely represents one of the methylene protons of the ethyleneamino linker. In the aromatic region, the broad peaks observed at 6.76, 6.91 and 7.20 ppm represent the characteristic salicylate aromatic ring protons. The integration of each proton signal closely matched the expected value.

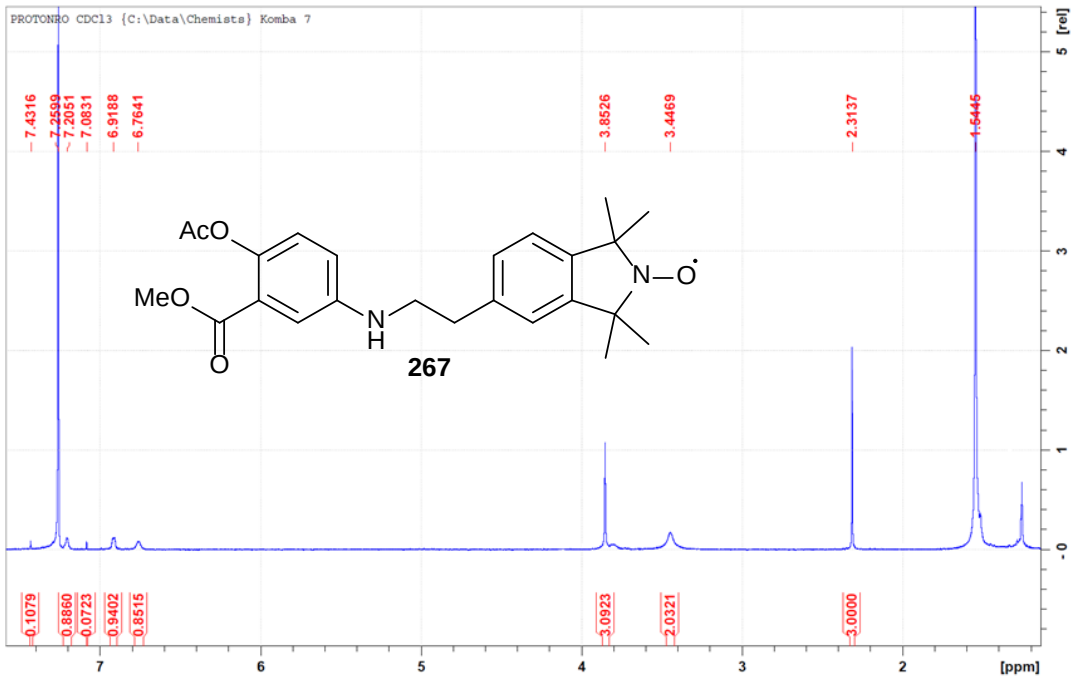


Figure 3.8. ^{1}H NMR (600 MHz) spectrum of 5-ethylamino-TMIO ester **267**.

The purity of compound **267** was verified by HPLC to be >95%. Mild basic hydrolysis of compound **267** afforded the target 5-(*N*-ethylamino-TMIO) salicylic acid conjugate **142** as a yellow solid in 72% yield. The identity of compound **142** was confirmed following analysis by HRMS and FTIR spectroscopy. A diagnostic mass of the $[M + H]^{+}$ ion was observed at m/z 370 in the HRMS spectrum. Characteristic strong broad OH and strong C=O absorptions at 2500-3500 and 1681 cm^{-1} respectively were observed in the FTIR spectrum.

Having successfully synthesized the targeted nitroxide-NSAID conjugates, their antioxidant and anti-inflammatory activities were assessed.

3.3.8 Antioxidant and Anti-inflammatory Effects of Selected Nitroxide-NSAID Conjugates

The compounds selected for preliminary biological tests are shown in **Figure 3.9** below. The results discussed below were obtained from preliminary studies conducted by collaborators in the Center for Cancer Research, a branch of National Cancer Institute, of the U.S-based National Institutes of Health (NIH).

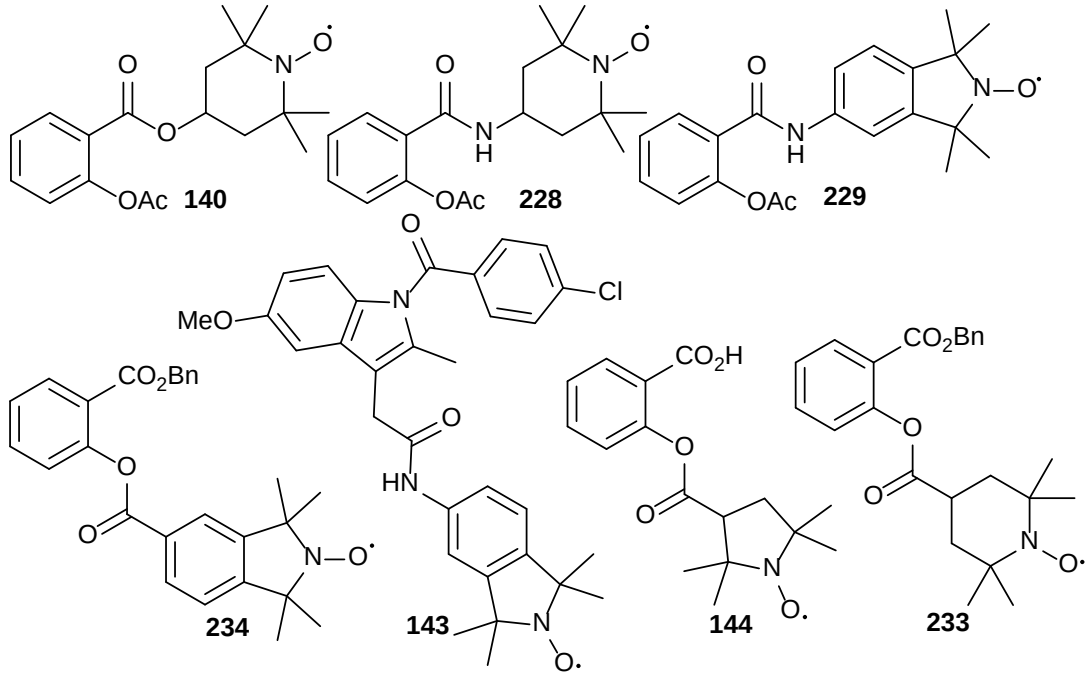
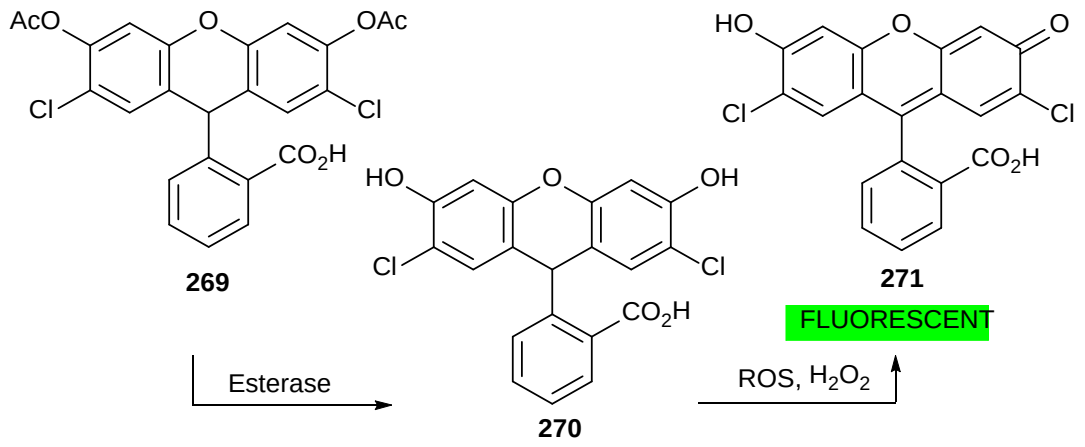


Figure 3.9. Selected nitroxide-NSAID conjugates for biological analysis.

3.3.8.1 Antioxidant Capacity of Nitroxide-NSAID Conjugate

The antioxidant capacity of the nitroxide-NSAID conjugates was determined by evaluating their ability to scavenge reactive oxygen species (ROS) generated by the Non-Small Cell Lung Cancer (NSCLC) cells, A549. NSCLC is a type of epithelial lung cancer relatively insensitive to chemotherapy and radiation therapy, and accounts for over 60% of lung cancers.[43] ROS production in A549 cells is initiated by the addition of hydrogen peroxide. The quantity of H_2O_2-induced ROS produced by A549 cells is estimated by employing 2′,7′-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA **269** is a cell-permeable non-fluorescent probe, which is de-esterified by cellular esterases to the polar, non-fluorescent derivative DCFH **270** (**Scheme 3.18**).[44-46]



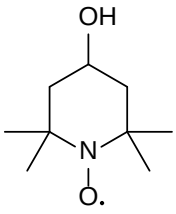
Scheme 3.18. ROS quantification by DCFH-DA probe: DCFH-DA **269** is deacetylated by cellular esterase to the nonfluorescent derivative DCFH **270**, which is in turn oxidized by ROS to the highly fluorescent DCF **271**.

In the presence of ROS, DCFH **270** is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein **271** (DCF), the intensity of which is correlated to the amount of ROS produced. Thus, A549 cells were placed in black 96 well plates, treated with 10 μM dichlorofluorescin diacetate for 1 hr and washed thoroughly. Some of the cells were treated 10 μM **140** or 100 μM TEMPOL **102**, and then 10 μM H_2O_2 added. Fluorescence measurements were taken at various times with excitation and emission wavelengths of 485 and 585 nm respectively. **Table 3.1** below shows that 5 min after addition of H_2O_2 addition to A549 cells, the ROS significantly increased to 194% of the basal fluorescence. TEMPOL **102** or **140** reduced the increase in ROS caused by H_2O_2 but had no effect on basal ROS levels. The results support the proposed strong antioxidant capacity of nitroxide-NSAID conjugate **140**. Notably, 10 μM of compound **140** displayed a similar ROS scavenging ability as 100 μM of TEMPOL **102** (the most studied antioxidant nitroxide).

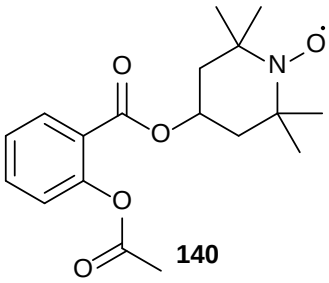
Table 3.1. ROS scavenging action of nitroxide-NSAID-conjugates determined by the DCFH-DA probe.

Addition	Relative fluorescence (%)
None	100 ± 5^{aa}
H_2O_2	$194 \pm 13^{**}$

H_2O_2 + **102** (100 μM)	144 ± 12*[a]
H_2O_2 + **140** (10 μM)	155 ± 15*[a]
102 (100 μM)	103 ± 6[aa]
140 (10 μM)	98 ± 4[aa]



102



140

* The mean value ± S.D. of 8 determinations is indicated; *, $p < 0.05$; ** $p < 0.01$ relative to control; a $p < 0.05$; aa $p < 0.01$ relative to H_2O_2. This experiment is representative of 2 others.

3.3.8.2 Anti-inflammatory Capacity of Nitroxide-NSAID Conjugates

To evaluate the anti-inflammatory capacity of the novel nitroxide-NSAID conjugates, studies were carried out using the A549/H1299 NSCLC cells. The target protein for this study was the epidermal growth factor receptor (EGFR). EGFR is a type of receptor tyrosine kinase (RTK) which is activated when ligands, such as epidermal growth factor (EGF), bind to it.[47] When activated, EGFR then facilitates the activation of multiple downstream signalling pathways within the cell, including the cell survival and proliferation pathways. EGFR is a membrane protein that is present in a range of cells including NSCLC. It facilitates the fast growth (proliferation) of NSCLC. Drugs such as gefitinib,[48] erlotinib, and afatinib, used to treat advanced NSCLS exert their therapeutic action by primarily blocking the EGFR signalling pathway.

Another downstream effect of the EGFR signalling pathway is that it induces COX-2 expression which in turn leads to the production of inflammation-related prostanoids such as PGE_2.[49-52] Increased COX-2-induced PGE_2 production is involved in various aspects of carcinogenesis. It is known to mediate inflammation response and can also enhance EGFR activity via a positive feedback loop.[49,52] Conventional

NSAIDs such as aspirin and indomethacin have displayed promising anti-carcinogenic effects by reducing PGE_2 production and inhibiting NSCLC cell growth.[49,53,54] Therefore, a rational drug design approach to NSCLC therapy may involve developing a multi-target drug that inhibits both EGFR and COX pathways, and reduces cellular oxidative stress.

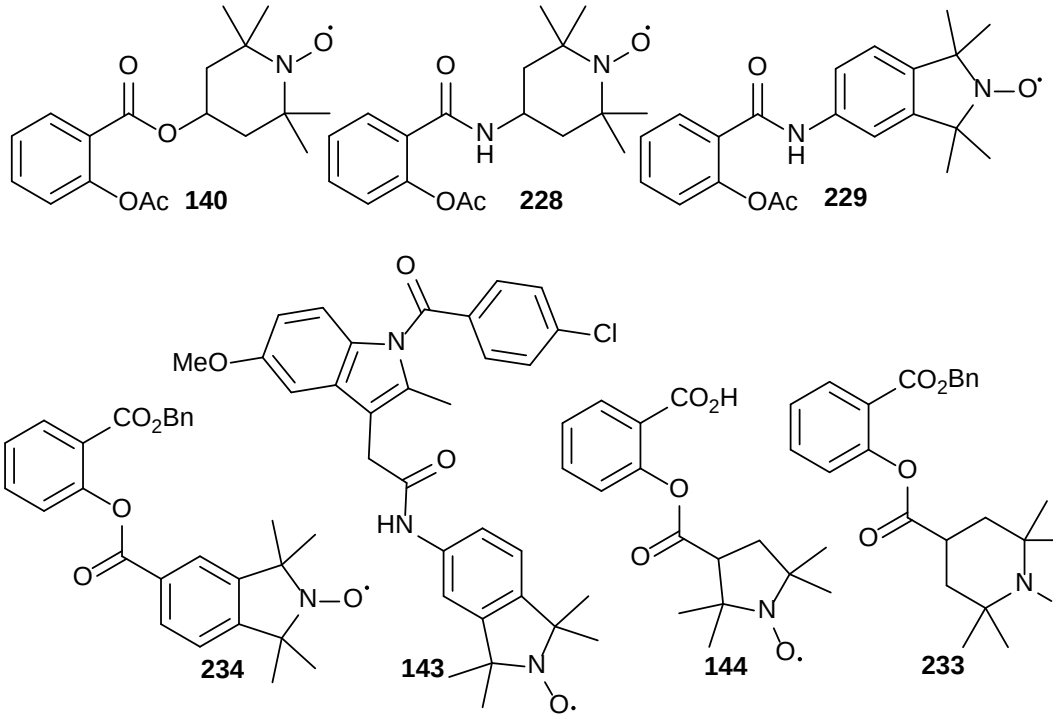
The novel nitroxide-NSAID conjugates were evaluated for their COX inhibitory action on NSCLC cells A549 or H1299. Briefly, selected nitroxide conjugates were dissolved in DMSO at a concentration of 30 μM and added to A549 or H1299 cells for 30 min. The COX reaction was started by the addition of 20 μg/mL of arachidonic acid at 37 ^{o}C for 5 min. The COX inhibition action of each compound was measured by the ELISA assay which was previously described in **Chapter 2**.

As shown in **Table 3.2** below, the ester-linked conjugates (**140**, **144**, **233** and **234**) displayed strong inhibitory capacity of the COX-induced PGE_2 production. In contrast, the amide-linked conjugates (**143**, **228** and **229**) showed moderate inhibitory action.

Table 3.2. Inhibitory effects of nitroxide-NSAID conjugates on COX-induced PGE_2 production.

Compound	Concentration (μM)	PGE_2 (pg/mL)
None		57 ± 4
140	30	31 ± 4*
143	30	58 ± 3
144	30	34 ± 6*

228	30	54 ± 6
229	30	45 ± 9
233	30	23 ± 5*
234	30	11 ± 1**

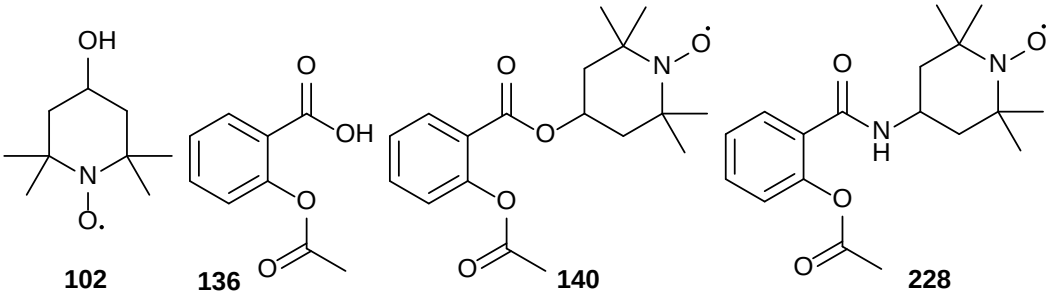


The mean value ± S.D. of 3 experiments each repeated in duplicate is shown; $p < 0.01$, **; $p < 0.05$, * using the student's t-test.

Further COX inhibition experiments were conducted at lower concentrations (4 μM) of selected conjugates (**140** and **228**) along with TEMPOL **102** and the parent aspirin **136**. As shown in **Table 3.3** below, the ester-linked conjugate **140** significantly inhibited PGE_2 productions at the lower concentration of 4 μM. The amide-linked conjugate **228** showed moderate inhibition at 4 μM. The most interesting result was the inhibitory action of the conjugates in comparison to parent aspirin **136.** Compound **140** was more potent at inhibiting COX-induced PGE_2 production in NSCLC cells than aspirin **136**.

Table 3.3. Inhibitory effects of nitroxide-NSAID conjugates on COX-induced PGE_2 production at lower concentration.

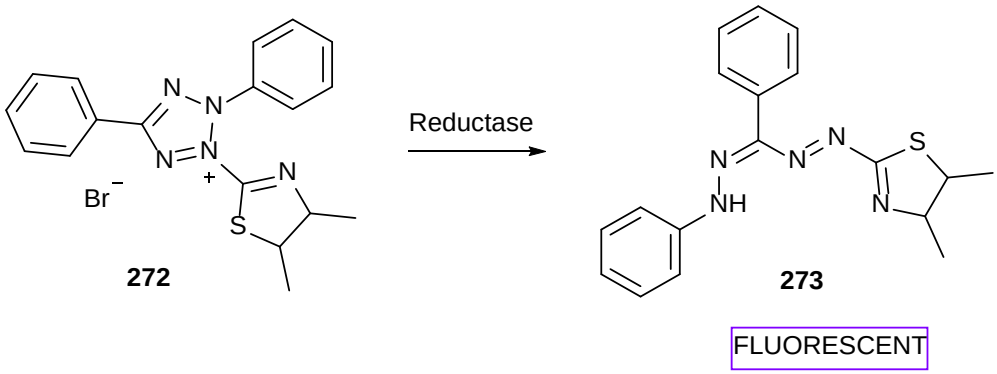
Compound	Concentration (µM)	PGE_2 (pg/mL)
None		126 ± 17
102	90	105 ± 11
136	90	79 ± 11*
102 + 136	90	75 ± 14*
140	4	87 ± 9*
228	4	99±13



102 **136** **140** **228**

The mean value ± S.D. of 4 determination is indicated using NCI-H1299 cells; $p < 0.05$, * using the Student's t-test. This experiment is representative of 2 others.

The nitroxide-NSAID conjugates were further tested for their inhibitory action on NSCLC proliferation using the MMT assay. MTT **272** is a yellow tetrazolium compound [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] that is reduced by various dehydrogenase enzymes to the dark blue MTT formazan **273** (**Scheme 3.19**).[55,56] The reduction, which involves the cleavage of the tetrazolium ring, only occurs in the mitochondria of living cells. Thus, the rate of cell proliferation, and conversely, the reduction in cell viability are directly related to quantity of MTT formazan produced. The formazan **273** is quantified by measuring its absorbance at certain wavelengths (usually between 500 and 600 nm).



Scheme 3.19. Mitochondrial reductase-facilitated reduction of MTT **272** to formazan **273**.

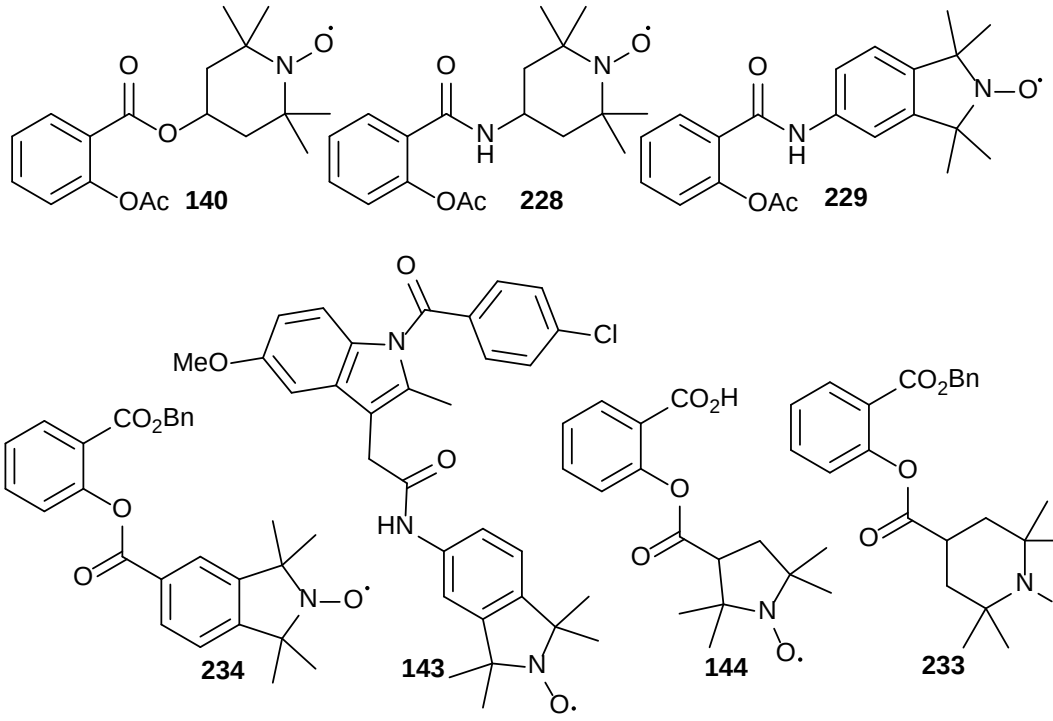
The nitroxide-NSAID conjugates were incubated with A549 cells for 3 days in 0.1 mL skin irritation test medium using 96 well plates. MTT (1 mg/mL, 15 µL) was added for 3 hours, followed by DMSO (150 µL) and the absorbance determined at 570 nm. The mean value ± S.D. of 8 determinations is indicated. This experiment is representative of 2 others.

As shown in **Table 3.4** below, the ester-linked conjugates (**140**, **144**, **233** and **234**) inhibited A549 cell proliferation with IC_{50} values in the range of 118-151 µM. In contrast, their amide-linked counterparts (**143**, **228** and **229**) displayed moderate cell inhibitory potency with IC_{50} values >300 µM).

Table 3.4. Inhibitory effects of nitroxide-NSAID conjugates on A549 cell proliferation.

Compound	IC_{50} (µM)
140	130 ± 23
143	>300
144	151 ± 8
228	>300
229	>300
233	121 ± 12

234	118 ± 16



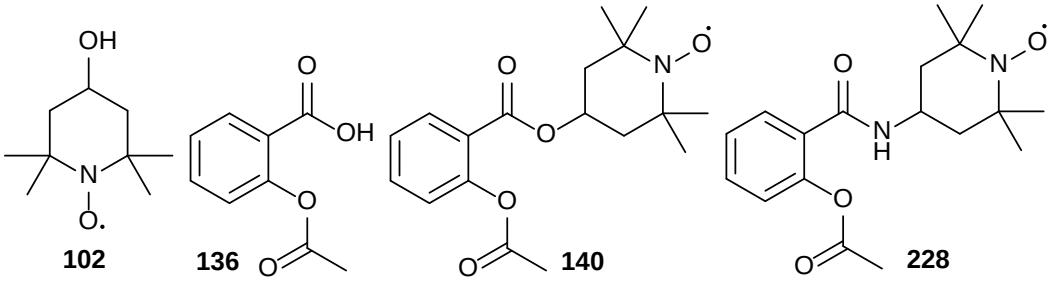
The mean value + S.D. of 8 determinations is indicated using A549 lung cancer cells; $p < 0.05$, *; $p < 0.01$, ** using the Student's t-test. This experiment is representative of 2 others.

Further A549 cell growth inhibitory studies were conducted with compound **140**, along with TEMPO **102** and aspirin **136**, at different concentrations. The results are shown in **Table 3.5** below. At a concentration of 180 µM, compound **140** displayed strong inhibitory capacity against the growth of A549 cells. However, no significant inhibition was observed for compound **140** at a lower concentration (18 µM). In contrast, TEMPOL **102** at 90 µM or 900 µM concentrations had little effect on A549 proliferation. Only a moderate inhibitory action was observed for aspirin **136**. However, this was only observed at higher aspirin **136** concentrations (900 µM).

Table 3.5. MMT assay results for nitroxide-NSAID conjugates inhibition of NSCLC A549 cell growth.

Compound	Concentration	Absorbance

	(μM)	(at 570 nm)
None		0.663 ± 0.037
102	90	0.666 ± 0.044
102	900	0.650 ± 0.060
136	90	0.682 ± 0.010
136	900	0.328 ± 0.021*
140	18	0.652 ± 0.021
140	180	0.089 ± 0.003*



The mean value + S.D. of 8 determinations is indicated using A549 lung cancer cells; $p < 0.05$, *; $p < 0.01$, ** using the Student's t-test. This experiment is representative of 2 others.

These results suggest that compound **140** is approximately an order of magnitude more potent at inhibiting NSCLC proliferation than aspirin **136**.

The ability of the nitroxide-NSAID conjugates to inhibit the growth of A549 cells in the MTT assay was also investigated in the presence of gefitinib. As mentioned earlier, gefitinib is an oral, selective anticancer agent known to reversibly inhibit the tyrosine kinase, an epidermal growth factor receptor (EGFR) that plays a key role in NSCLC carcinogenesis.[57-59] It was anticipated that adding gefitinib to the nitroxide-NSAID conjugates would produce a synergistic inhibitory action on NSCLC cell growth. Thus, compound **140**, in the presence of gefitinib, was analyzed with the MTT assay. As shown in **Figure 3.10** below, compound **140** alone had half-maximum inhibitory action on the proliferation of A549 cells with an IC_{50} value of 100 μM.

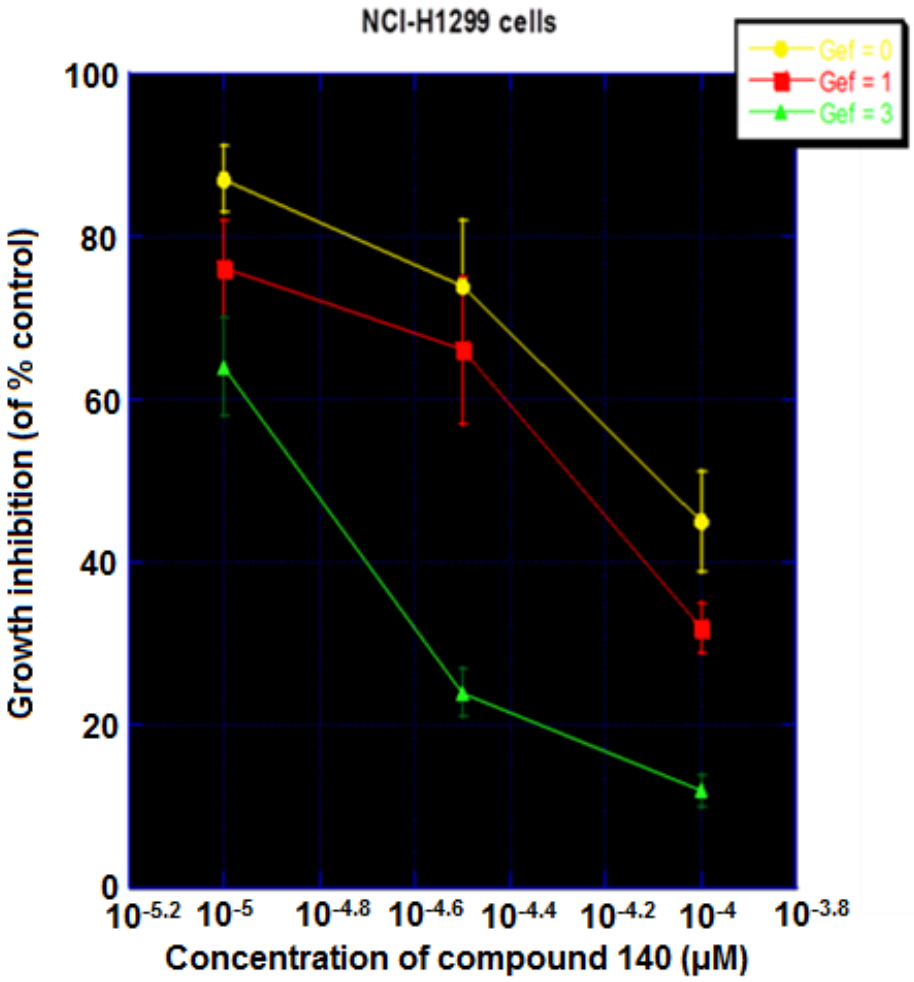


Figure 3.10. MTT assay for the inhibitory effect of nitroxide conjugate **140** (either alone or in combination with gefitinib) on NSCLC H1299 cell proliferation. The inhibition of cell growth was determined when gefitinib was 0 (●), 1 μg/mL (■) and 3 μg/mL (▲). The mean value ± S.D. of 8 determinations is indicated.

Upon the addition of 3 μg/mL of gefitinib, compound **140** displayed significant inhibitory action with an IC_{50} value 30 μM. Gefitinib is however known to possess a dose-dependent antiproliferation effects on H1299 cells with an IC_{50} value 40 μM.[60]

To further evaluate the additive effect of compound **140** with gefitinib, a clonogenic assay was conducted for A549 cells. The clonogenic assay is an *in vitro*-based cell survival assay that tests the ability of a cell to proliferate indefinitely and form a large colony.[61,62] It is commonly used to determine the ability of a cell to retain its unlimited growth after treatment with an agent that inflicts insults on the colony.

Thus, large colonies of A549 cells were formed using a clonogenic cell survival assay (**Figure 3.11 A**).

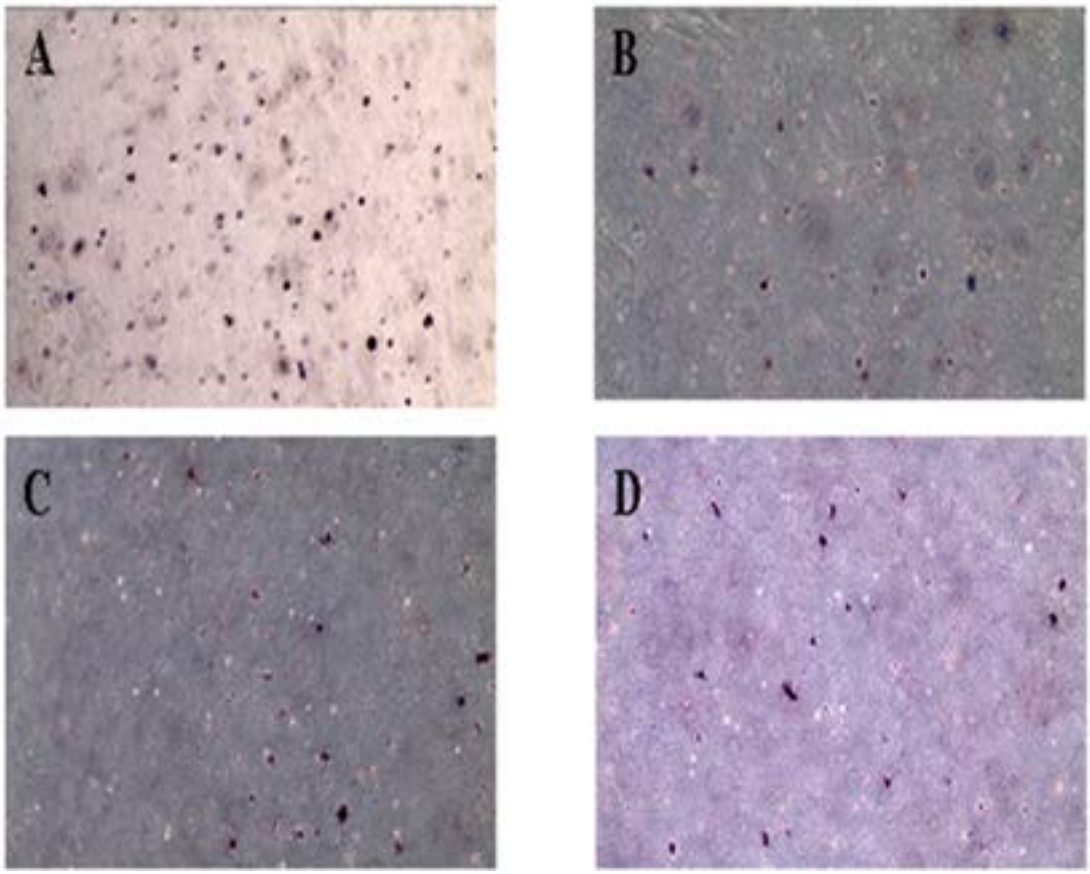


Figure 3.11. Clonogenic assay results for the inhibitory effect of nitroxide-NSAID conjugate **140** on A549 cells growth. Colonies of A549 are shown as a function of (**A**) untreated controls, (**B**) 10 μM **140**, (**C**) 1 μg/mL gefitinib and (**D**) 10 μM **140** plus 1 μg/mL gefitinib.

After the addition of 10 μM of compound **140** or 1 μg/mL of gefitinib, the number and size of the colonies were markedly reduced (**Figure 3.11 B** and **C**). In the presence of gefitinib and **140**, few colonies were present (**Figure 3.11 D**). These results suggest that gefitinib and the nitroxide-NSAID conjugates work synergistically to inhibit NSCLC growth.

Due to such promising preliminary results obtained for the selected conjugates, more antioxidant and anti-inflammatory studies are currently being carried out with the rest of the novel compounds.

3.4 Summary of Results

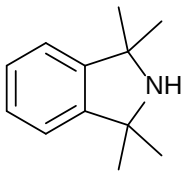
Using the pharmacophore hybridization strategy, a series of novel potential dual-acting (antioxidant and anti-inflammatory) nitroxide-NSAID conjugates were designed and synthesized. In general, the synthesis involved carbodiimide-facilitated coupling of various nitroxide compounds with a number of NSAIDs. The parent NSAID templates employed included indomethacin, aspirin, salicylic acid, and 5-amino salicylic acid. Although the pyrrolidine and piperidines nitroxide precursors are commercially available, each isoindoline nitroxide compound was readily synthesized. In the synthesis of the water-soluble antioxidant nitroxide, CTMIO, three new alternative synthetic routes were developed. Though each new route had a modest overall yield (16-18%), the formyl route was the quickest. For the synthesis of the AAD-2004 nitroxide analogue **142**, the key step involved selective reduction of the amide group of **251**. This was successfully carried out using an *in situ*-generated sodium acyloxyborohydride as the active reducing agent under refluxing reaction conditions.

Nine novel nitroxide-NSAID conjugates were evaluated for their antioxidant and anti-inflammatory effects on the A549 Non-Small Cell Lung Cancer cells. Some of the nitroxide conjugates displayed significant antioxidant effect on ROS generated by A549 cells. Notably, the nitroxide conjugate **140** showed a better ROS scavenging activity than the versatile antioxidant, TEMPOL **102**. Some of the novel nitroxide conjugates displayed promising anti-inflammatory action by inhibiting COX-induced PGE_2 production by A549 cells. While the ester-linked conjugates (**140**, **144**, **233** and **234**) possessed strong inhibitory effects on the COX enzyme, the amide-linked counterparts (**143**, **228** and **229**) displayed only moderate inhibitory effect. Notably, the nitroxide conjugate **140** possessed better inhibitory effect on the COX enzyme than parent aspirin **136**. In addition to the antioxidant and anti-inflammatory effects, compound **140** was shown to inhibit the proliferation of A549 NSCLC cells. Clonogenic assay studies of A549 cells indicated that combining the conjugate **140** with anticancer agent gefitinib produced a synergistic effect on inhibiting A549 cells growth. The anticancer effect of compound **140** on A549 cell growth is quite promising and further studies are currently being undertaken.

Another prominent class of drugs in which chronic inflammation and oxidative stress play a key role in the pathogenesis and the associated drug-induced cellular damage is the sympathomimetic stimulant class of drugs. Due to such noxious effects, long-term therapeutic benefits of drugs such as such as L-Dopa **147** and Ritalin **148** are limited. L-Dopa **147** and Ritalin **148** are the main therapeutic agents used to treat Parkinson's disease and ADHD respectively. The next chapter discusses the use of the pharmacophore hybridization approach to synthesize potential hybrid dual-acting stimulants by combining antioxidant nitroxides with L-Dopa and Ritalin.

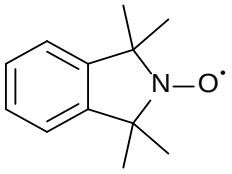
3.5 Experimental

3.5.1. Synthesis of 1,1,3,3-Tetramethylisoindoline 207



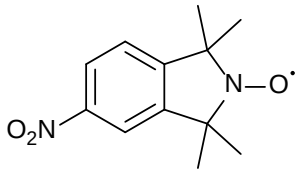
A reaction mixture of *N*-benzyl-1,1,3,3-tetramethylisoindoline **169** (5 g, 18.84 mmol, 1 equiv.) and Pd/C (500 mg, 10% wt.) in glacial acetic acid (50 mL) was shaken under hydrogen (50 psi) in a Parr Hydrogenator for 4 h. The mixture was then concentrated under reduced pressure and basified with NaOH (2 M, to pH 12). The aqueous layer was extracted with Et_2O (100 mL x 4) and the combined Et_2O extracts were washed with brine (60 mL x 2), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give 1,1,3,3-tetramethylisoindoline **207** as a beige solid (3.17 g, 96%), which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.48 (s, 12 H, C*H*$_3$), 1.9 (s, 1 H, N-H), 7.15 (m, 2 H, Ar-*H*), 7.27 (m, 2 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.9 (C-*C*H$_3$), 62.8 (*C*-CH$_3$), 121.4 (Ar-*C*), 127.1 (Ar-*C*), 148.8 (Ar-*C*).

3.5.2. Synthesis of 1,1,3,3-Tetramethylisoindolin-2-yloxyl 59



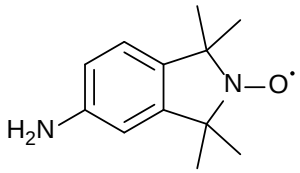
*m*CPBA (6.16 g, 22.25 mmol, 1.3 equiv.) was added to a solution of 1,1,3,3-tetramethylisoindoline **207** (3 g, 17.1 mmol, 1 equiv.) in DCM (200 mL) at 0 ^{o}C. The cooling bath was removed after 30 min and the reaction stirred at RT for further 2 h. The DCM layer was washed with HCl (2 M, 50 mL), NaOH (2 M, 50 mL x 2), and brine solutions (50 mL) and before being dried over anhydrous Na_2SO_4. The DCM was removed under reduced pressure and the residue obtained was purified by a short silica gel column chromatography (Hexane/DCM, 1:1) to give 1,1,3,3-tetramethylisindolin-2-yloxyl **59** as yellow solid (3.058 g, 94%). Mp. 128-129 ^{o}C (Lit.,[63] 128-129 ^{o}C).

3.5.3. Synthesis of 5-Nitro-1,1,3,3-tetramethylisoindolin-2-yloxyl 29



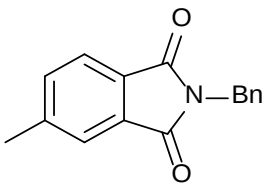
1,1,3,3-Tetramethylisoindolin-2-yloxyl **59** (2 g, 1.05 mmol) was dissolved in H_2SO_4 (15 mL, 98%) and cooled to 0 °C. Nitric acid (3.75 mL, 70%) was added dropwise and the reaction mixture was stirred at 0 °C for 1 h. The cooling bath was removed and stirring was continued for 4 h. The reaction mixture was cooled to 0 °C, diluted by careful addition ice/H_2O and then basified with NaOH solution (10 M). The reaction mixture was extracted with Et_2O (60 mL x 4) and the combined organic extracts were washed with brine (60 mL), dried with anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The residue was purified by filtration through a short silica gel column (EtOAc) and recrystallized from EtOH to afford bright orange prisms of 5-nitro-1,1,3,3-tetramethylisoindolin-2-yloxyl **29** (2 g, 81%). Mp. 161-162 °C (lit.,[8] 160-162°C).

3.5.4. Synthesis of 5-Amino-1,1,3,3-tetramethylisoindolin-2-yloxyl 108



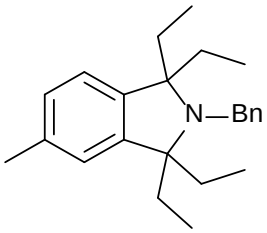
A reaction mixture of 5-nitro-1,1,3,3-tetramethylisoindolin-2-yloxyl **29** (1.5 g, 6.38 mmol, 1 equiv) and Pd/C (150 mg, 10% wt.) in MeOH (25 mL) was stirred under hydrogen (50 psi) in a Parr Hydrogenator for 4 h. The mixture was then shaken for further 20 min in open air in the presence of PbO_2 (19 mg, 80 µmol, 0.0125 equiv.). The mixture was then filtered through Celite and concentrated under reduced pressure. The crude residue was recrystallized from EtOH to give 5-amino-1,1,3,3-tetramethylisoindolin-2-yloxyl **108** as yellow solid (1.15 g, 88%). Mp. 196-198 °C (Lit.,[64] 198 °C).

3.5.5. Synthesis of N-Benzyl-5-methylphthalimide 210



4-Methylphthalic anhydride **209** (10 g, 61.70 mmol, 1 equiv.) was dissolved in glacial acetic acid (50 mL). To this was added benzylamine (10 mL, 92.60 mmol, 1.5 equiv.) and the resulting solution refluxed for 1 hr. The reaction mixture was cooled to room temperature, poured onto ice/water (150 mL) and stirred to give a white precipitate which was then filtered and recrystallized from ethanol to give fluffy white crystals of *N*-benzyl-5-methylphthalimide **210** (15.21 g, 98%). Mp. 128-130 ^{o}C (Lit.,[10] 130 ^{o}C). ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 2.50 (s, 3H, CH_3), 4.83 (s, 2H, CH_2), 7.26-7.33 (m, 3H, Ar-H).

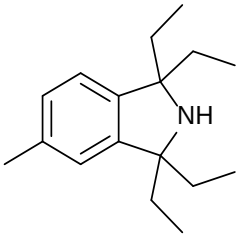
3.5.6. Synthesis of N-Benzyl-1,1,3,3-tetraethyl-5-methyl isoindoline 211



A solution of *N*-benzyl-5-methylphthalimide **210** (20 g, 80 mmol, 1 equiv.) in anhydrous toluene (200 mL) was added to a freshly prepared with ethylmagnesium iodide [formed from ethyl iodide (38.4 mL, 480 mmol, 6 equiv.) and magnesium

turnings (23.36 g, 940 mmol, 12 equiv.) in Et_2O (200 mL)]. The Et_2O was distilled off using a Dean–Stark and the reaction mixture was refluxed for 4 h and then allowed to cool to RT. The mixture was diluted with hexane and filtered through Celite and washed thoroughly with extra hexane. Air was bubbled through the filtrate overnight and the crude residue was passed through a short column of basic alumina and concentrated *in vacuo* to afford *N*-benzyl-1,1,3,3-tetraethyl-5-methylisoindoline **211** as a colourless oil (9 g, 34%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.78 (t, *J* = 7.36, 3.04, 12 H, C*H*$_3$), 1.45–1.62 (m, 4 H, C*H*$_2$), 1.85–1.95 (m, 4 H, C*H*$_2$), 2.37 (s, 3 H, C*H*$_3$), 4 (s, 2 H, C*H*$_2$), 6.86 (s, 1 H, Ar-*H*), 6.94 (d, 1 H, *J* = 7.7 Hz, Ar-*H*), 7.02 (d, 1 H, *J* = 7.7 Hz, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 9.59 (CH_3), 9.62 (CH_3), 21.5 (CH_3), 30.32 (CH_2), 30.34 (CH_2), 46.8 (CH_2), 71.0 (*C*CH_3), 71.2 (*C*CH_3), 123.1 (Ar-*C*), 123.9 (Ar-*C*), 126.47 (Ar-*C*), 126.48 (Ar-*C*), 127.7 (Ar-*C*), 129.2 (Ar-*C*), 135.0 (Ar-*C*), 141.7 (Ar-*C*), 142.5 (Ar-*C*), 144.7 (Ar-*C*) ppm.

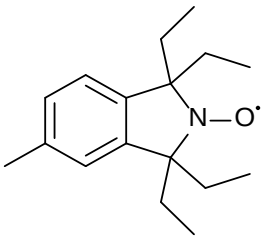
3.5.7. Synthesis of 1,1,3,3-Tetraethyl-5-methylisoindoline 212



A reaction mixture of *N*-benzyl-1,1,3,3-tetraethyl-5-methylisoindoline **211** (5 g, 14.91 mmol, 1 equiv.) and Pd/C (500 mg, 10% wt.) in glacial acetic acid (50 mL) was shaken under hydrogen (50 psi) in a Parr Hydrogenator for 4 h. The mixture was then concentrated under reduced pressure and basified with NaOH (2 M, to pH 12). The aqueous layer was extracted with Et_2O (100 mL x 4) and the combined Et_2O extracts were washed with brine (50 mL x 2), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give 1,1,3,3-tetraethyl-5-methylisoindoline **212** (3.51 g, 96%), which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.9 (m, 12 H, CH_2CH_3), 1.7 (m, 8 H, C*H*$_2$), 2.38 (s, 3 H, Ar-C*H*$_3$), 6.89 (s, 1 H, Ar-*H*), 6.98 (d, 1 H, Ar-*H*), 7.05 (d, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 9.0 (*C*H_3), 21.4 (Ar-*C*H_3), 33.78 (*C*H_2), 33.79 (*C*H_2), 68.0

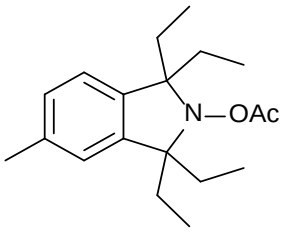
($C_{quat.}$), 68.2 (C_{quat}), 122.2 (Ar-*C*), 123.1 (Ar-*C*), 127.4 (Ar-*C*), 136.1 (Ar-*C*), 144.7 (Ar-*C*), 147.7 (Ar-*C*).

3.5.8. Synthesis of 1,1,3,3-Tetraethyl-5-methylisoindolin-2-yloxyl 213



m-Chloroperoxybenzoic acid (4.4 g, 15.89 mmol, 1.3 equiv.) was added to a solution of 1,1,3,3-tetraethyl-5-methylisoindoline **212** (3 g, 12.22 mmol, 1 equiv.) in DCM (250 mL) at 0 ^{o}C. The cooling bath was removed after 30 min and the reaction stirred at RT for further 2 h. The DCM layer was washed with HCl (2 M, 50 mL), NaOH (2 M, 50 mL x 3), and brine (50 mL x 2) solutions and before being dried over anhydrous Na_2SO_4. The DCM was removed under reduced pressure and the residue obtained was purified by a short silica gel column chromatography (Hexane/DCM, 1:1) to give 1,1,3,3-tetraethyl-5-methylisoindolin-2-yloxyl **213** as yellow solid (2.67 g, 84%). HRMS (ES): *m/z* (%) = 261.2049 (10) $[M + H]^+$; calcd. for $C_{17}H_{26}NO^{\bullet}$: 260.2014; found 260.2015.

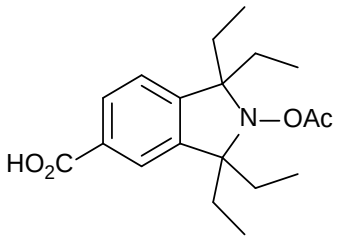
3.5.9. Synthesis of 2-Acetoxy-1,1,3,3-tetraethyl-5-methyl isoindoline 214



A reaction mixture of 1,1,3,3-tetraethyl-5-methylisoindolin-2-yloxyl **213** (1 g, 3.84 mmol, 1 equiv.) and Pd/C (100 mg, 10% wt.) in THF (25 mL) was flushed with Ar for 10 min. Then, a balloon containing H_2 was connected and the reaction mixture stirred for 20 min. The reaction mixture was cooled in ice/H_2O bath followed by the addition of TEA (1.1 mL, 7.68 mmol, 2 equiv.) and then AcCl (545 µL, 7.68 mmol, 2 equiv.) dropwise. The cooling bath was removed after 30 min and stirring was

continued for further 1 h. The reaction mixture was filtered through Celite and concentrated *in vacuo*. The crude residue was dissolved in EtOAc (150 mL) and washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification of the crude product by silica gel column chromatography (Hexane/EtOAc, 5:1) gave 2-acetoxy-1,1,3,3-tetraethyl-5-methylisoindoline **214** as a clear solid (1.06 mg, 91%). Mp. 76–78 ^{o}C (Lit.,[10] 76–78 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.79 (t, 6 H, CH_2CH_3), 0.96 (t, 6 H, CH_2CH_3), 1.62 (m, 2 H, CH_2), 1.75 (m, 2 H, CH_2), 1.86 (m, 2 H, CH_2), 1.96 (m, 2 H, CH_2), 2.1 (s, 3 H, CH_3), 2.37 (s, 3 H, CH_3), 6.86 (s, 1 H, Ar-*H*), 6.94 (d, 1 H, Ar-*H*), 7.06 (dd, 1 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 8.6 (CH_3), 9.4 (CH_3), 19.4 (CH_3), 21.6 (CH_3), 28.9 (CH_2), 30.3 (CH_2), 73.5 (CCH_2), 73.6 (CCH_2), 123.4 (Ar-*C*), 124.0 (Ar-*C*), 127.5 (Ar-*C*), 136.2 (Ar-*C*), 136.7 (Ar-*C*), 138.8 (Ar-*C*), 141.8 (Ar-*C*), 170.5 (*C*=O) ppm. ATR-FTIR: υ_{max}= 2956 (m, Ar C-H), 1770 (s, C=O), 1190 (s, C-O) cm^{-1}.

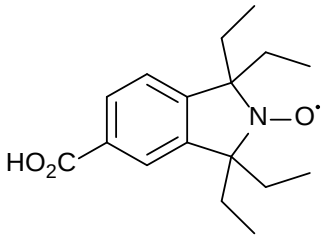
3.5.10. Synthesis of 2-Acetoxy-5-carboxy-1,1,3,3-tetraethylisoindoline 215



$MgSO_4$ (169 mg, 1.65 mmol, 0.5 equiv) and freshly prepared $KMnO_4$ solution (32.7 mL, 13.2 mmol, 4 equiv, 0.4 M) were added to a solution of *N*-acetoxy-1,1,3,3-tetraethyl-5-methylisoindoline **214** (1 g, 3.3 mmol, 1 equiv) in *tert*-butyl alcohol (20 mL) and the resulting reaction mixture was stirred at 70 ^{o}C for 1 d. The reaction mixture was cooled to RT, treated with *i*PrOH (10 mL) and stirred overnight. The mixture was filtered through Celite and the solvent removed *in vacuo*. The resulting residue was picked up in EtOAc (150 mL), washed with water and brine (40 mL), dried over anhydrous Na_2SO_4 and the filtrate concentrated *in vacuo*. Purification of the resulting residue by silica gel flash chromatography (eluent: initial: (DCM/EtOAc, 3:2) gave 2-acetoxy-5-carboxy-1,1,3,3-tetraethylisoindoline **215** as a white solid (660 mg, 60%). Mp. 168–170 °C (Lit.,[10] 168–170 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.8 (t, 6 H, CH_3), 0.97 (t, 6 H, CH_3), 1.78 (m, 4 H, CH_2), 2 (m, 4 H,

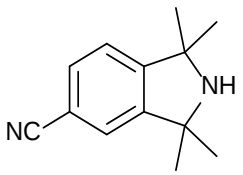
C*H*$_2$), 2.12 (s, 3 H, C*H*$_3$), 7.17 (d, 1 H, Ar-*H*), 7.81 (1 H, Ar-*H*), 8.03 (d, 1 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 8.5 (*C*H$_3$), 9.4 (*C*H$_3$), 19.3 (*C*H$_3$), 28.9 (*C*H$_2$), 30.2 (*C*H$_2$), 73.7 (*C*CH$_3$), 74.0 (*C*CH$_3$), 123.7 (Ar-*C*), 125.4 (Ar-*C*), 128.0 (Ar-*C*), 128.9 (Ar-*C*), 142.3 (C_{quat}), 148.3 (Ar-*C*), 170.3 (*C*=O), 172.1 (*C*=O) ppm.

3.5.11. Synthesis of 5-Carboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl 206



A suspension of 2-acetoxy-5-carboxy-1,1,3,3-tetraethylisoindoline **215** (500 mg, 1.5 mmol, 1 equiv.) in H_2O/MeOH (5 mL/5 mL) was cooled to 0 ^oC. LiOH (182.6 mg, 7.5 mmol, 5 equiv.) was added and the reaction mixture was stirred overnight while allowed to warm to RT. The resulting solution was diluted with H_2O (20 mL), washed with Et_2O (20 mL x 2) and the Et_2O layer discarded. The aqueous layer was cooled in ice bath, acidified with HCl (2 M) and then extracted with Et_2O (30 mL x 4). The combined Et_2O extracts were stirred over PbO_2 (90.5 mg, 375 µmol, 0.25 equiv.) for 20 min, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude residue was recrystallized from MeCN to give 5-carboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl **206** as yellow crystals (362 mg, 83%). Mp. 97-99 ^oC (Lit.,[10] 97-99 ^oC).

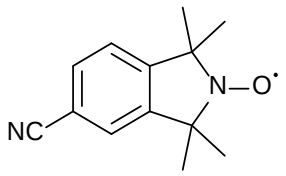
3.5.12. Synthesis of 5-Cyano-1,1,3,3-tetramethylisoindoline 71



A Schlenk vessel that contained a mixture of 5-bromo-1,1,3,3-tetramethylisoindoline **66** (500 mg, 1.97 mmol, 1 equiv.), $K_4[Fe(CN)_6]$ (207.5 mg, 984 µmol, 0.25 equiv.), CuI (45 mg, 472 µmol, 0.12 equiv.) *N*-butylimidazole (825 µL, 3.934 mmol, 2 equiv.) in *o*-xylene (5 mL) was degassed and then refluxed at 180 ^oC for 4 d. The

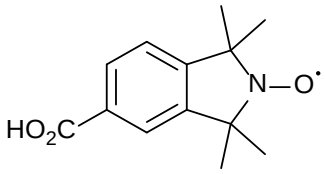
resulting mixture was allowed to return to RT before it was diluted with water and then extracted with EtOAc (60 mL x 4). The combined EtOAc extracts were washed with brine (50 mL) and dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc) to give **71** as a mixture with the *N*-butylimidazole complex (458 mg) which was used in subsequent steps without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.46 (s, 12 H, 4 x C*H*$_3$), 1.71 (br. s, 1 H, N*H*), 7.20 (d, 1 H, *J* = 7.6 Hz, Ar-*H*), 7.40 (s, 1 H, *J* = 7.6 Hz, Ar-*H*), 7.53 (d, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 31.6 (*C*H$_3$), 31.8 (*C*H$_3$), 62.8 (C_{quat}), 63.2 (C_{quat}), 111.0 (C_{quat}N), 119.3 (Ar-*C*), 122.5 (Ar-*C*), 125.6 (Ar-*C*), 131.5 (Ar-*C*), 150.1 (Ar-*C*), 154.2 (Ar-*C*). HRMS (ES): *m*/*z* (%) = 201 (100, M+H); Calcd. for $C_{13}H_{16}N_2$ [M + H]$^+$: 201.1313; found 201.1317. ATR-FTIR: υ_{max}= 3321 (w, N-*H*), 2226 (m, s, C≡N).

3.5.13. Synthesis of 5-Cyano-1,1,3,3-tetramethylisoindol-2-yloxyl 217



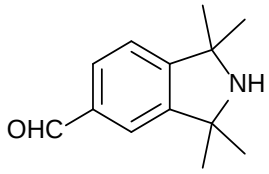
m-Chloroperoxybenzoic acid (864 mg, 2.96 mmol, 1.3 equiv., 77 %) was added to an ice-cold solution of the crude 5-cyano-1,1,3,3-tetramethylisoindoline **71** (458 mg, 2.28 mmol, 1.0 equiv.) in DCM (200 mL). The resulting solution was stirred for 1 h at 0 °C. The cooling bath was removed and stirring was continued at RT for a further 1.5 h with additional dichloromethane added to dissolve the formed precipitate. The bright-yellow organic phase was washed successively with HCl (0.5 M (50 mL), NaOH (2 M (50 mL) and brine (50 mL) solutions and dried with anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the resultant solid recrystallized from cyclohexane to afford 5-cyano-1,1,3,3-tetramethylisoindol-2-yloxyl **217** as a bright-yellow solid (330 mg, 78 % from **66**). Mp. 127 °C. FTIR (ATR): $\tilde{\nu}_{max}$ = 2227 (m, C≡N) cm^{-1}. HRMS (ES): *m*/*z* (%) = 217 (80) [M + 2H]$^+$; calcd. for $C_{13}H_{16}N_2O$ [M + 2H]$^+$ 217.1341; found 217.1281.

3.5.14. Synthesis of CTMIO 70 from 5-Cyano-1,1,3,3-tetramethylisoindol-2-yloxyl 217



A suspension of 5-cyano-1,1,3,3-tetramethylisoindol-2-yloxyl **217** (150 mg, 698 µmol, 1.0 equiv.) in NaOH (3 mL, 2 M aqueous solution) and EtOH (0.6 mL) was heated at reflux for 16 h. The reaction was cooled to room temperature, diluted with water (5 mL), washed with Et_2O (3 × 10 mL) and then acidified to pH 1 with HCl (2 M aqueous solution). The acidic aqueous layer was extracted with Et_2O (4 × 20 mL) and the combined organic layers washed with brine (15 mL), dried with anhydrous Na_2SO_4 and filtered. The filtrate was concentrated *in vacuo* and recrystallized from MeCN to obtain CTMIO **70** as a yellow solid (155 mg, 95 %). Mp. 211 °C (Lit.,[14] 214–218 °C).

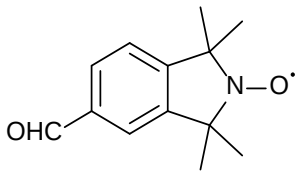
3.5.15. Synthesis of 5-Formyl-1,1,3,3-tetramethylisoindoline 73



n-Butyllithium (1.6 M in hexanes, 5.45 mL, 8.72 mmol, 2.2 equiv.) was added dropwise to a solution of 5-bromo-1,1,3,3-tetramethylisoindoline **66** (1 g, 3.94 mmol) in dry THF (11 mL) at -78°C under argon. After 10 minutes the reaction was quenched by the addition of DMF (0.915 mL, 11.8 mmol, 3 equiv.). The resulting mixture was allowed to warm to room temperature and then diluted with H_2O. The aqueous phase was acidified with HCl (2 M) and extracted with Et_2O (40 mL x 4) to remove non-basic impurities. The aqueous phase was then basified (pH >10) with NaOH (5 M) and extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (0.2% MeOH/$CHCl_3$) to afford **73** (800 mg, 100%) as an off-white

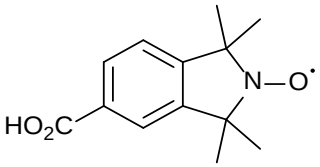
crystalline solid. ^{1}H NMR ($CDCl_3$): δ = 1.50 (s, 6 H, 2 x C*H*$_3$), 1.51 (s, 6 H, 2 x C*H*$_3$), 7.29 (d, 1 H, *J* = 7.8 Hz, Ar-*H*), 7.68 (d, 1 H, *J* = 2.2 Hz, Ar-*H*), 7.80 (dd, 1 H, *J* = 7.8 Hz, 2.2 Hz, Ar-*H*), 10.03 (s, 1 H, O=C*H*).

3.5.16. Synthesis of 5-Formyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 27



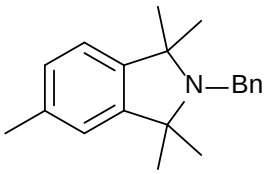
A solution of *m*CPBA (1.483 g, 5.09 mmol, 1.1 equiv., 77%) in DCM (10 mL) was added dropwise to a solution of 5-formyl-1,1,3,3-tetramethylisoindoline **73** (1 g, 4.62 mmol) in DCM (200 mL) at 0 ^{o}C. The reaction mixture was stirred for 2 h the resulting bright yellow solution was washed with dilute HCl (0.5 M, 20 mL), NaOH (2 M, 20 mL) and brine (20 mL) solutions and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the residue purified by flash column chromatography (Hexane/EtOAc, 4:1) to give compound **27** as a bright yellow solid (928 mg, 92%). Mp. 138-140°C (Lit.,[13] 139-141°C).

3.5.17. Synthesis of CTMIO 70 from 5-Formyl-TMIO 27



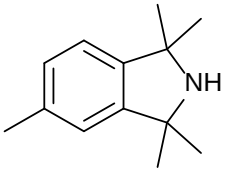
NaOH (29.3 mg, 733 μmol, 4.0 equiv.) and $AgNO_3$ (62.3 mg, 366.5 μmol, 2.0 equiv.) were added to water (3 mL) and stirred at room temperature for 30 min. A solution of 5-formyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **27** (40 mg, 183.3 μmol, 1 equiv.) in ethanol (1.5 mL) was added to the resulting mixture and the reaction mixture stirred for 6 h. The solution was acidified (pH < 2) by careful addition of HCl (2 M aqueous solution). The aqueous layer was extracted with Et_2O (3 × 20 mL) and the combined ether layers were washed with brine (15 mL), dried with anhydrous Na_2SO_4 and the filtrate concentrated *in vacuo* and recrystallized from MeCN to give **70** as a yellow solid (31 mg, 72 %). Mp. 211 °C (Lit.,[14] 214–218 °C).

3.5.18. Synthesis of N-Benzyl-1,1,3,3,5-pentamethylisoindoline 218



A solution of *N*-benzyl-5-methylphthalimide **209** (55.834 g, 222.2 mmol, 1 equiv) in dry toluene (500 mL) was added slowly to a freshly prepared methylmagnesium iodide solution [formed from methyl iodide (83 mL, 1.33 mol, 6 equiv) and magnesium turnings (40 g, 1.55 mol, 7 equiv)] in dry Et_2O. The Et_2O was distilled off ether through a Dean Stalk and the reaction mixture refluxed at 110 ^oC for 4 h. The mixture was cooled to RT, filtered through Celite and washed several times with excess hexane. Air was bubbled through the filtrate overnight and the resulting residue filtered through a short basic alumina column (eluent: Hexane) to separate product from the purple intractable material. Concentration *in vacuo* afforded compound *N*-benzyl-1,1,3,3,5-pentamethylisoindoline **218** as a white solid which was readily recrystallized from methanol or pentane (15.67 g, 34%). Mp. 58-59 ^oC. HPLC purity (>95%). ^1H NMR (400 MHz, $CDCl_3$): = δ 1.30 (s, 6 H, C*H*$_3$),1.31 (s, 6 H, C*H*$_3$), 2.39 (s, 3 H, C_5-C*H*$_3$), 4.00 (s, 2 H, C*H*$_2$), 6.96 (s, 1H, C_4-*H*), 7.06 (q, 2 H, *J* = 7.68, Ar-H), 7.20 - 7.25 (m, 1 H, Ar-*H*), 7.27 - 7.32 (m, 3 *H*, Ar-*H*), 7.48 (d, 2 H *J* = 7.37 Hz, Ar- *H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.4 (Ar-*C*H_3), 28.4 (*C*H_3), 28.5(*C*H_3), 46.3 (*C*H_2), 64.9 (C_{quat}), 65.1 (C_{quat}), 121.1 (Ar-*C*), 121.9 (Ar-*C*), 126.4-128.3 (Ar-*C*H, 3 C), 136.4 (Ar-C_{quat}), 143.5 (Ar-C_{quat}'), 145.1 (Ar-C_{quat}).148.0 (Ar-C_{quat}). HRMS (ES): *m*/*z* (%) = 180 (100, M+H), 269/71 (48/46) $[M+2H]^+$. Calc. for $C_{20}H_{25}N$ $[M]^+$: 179.1980; found 179.1987. ATR-FTIR: υ_{max}= 3290 (w, Ar C-H).

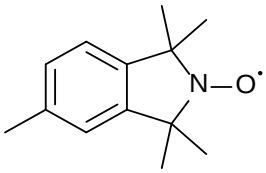
3.5.19. Synthesis of 1,1,3,3,5-Pentamethylisoindoline 219



A mixture of *N*-benzyl-1,1,3,3,5-pentamethylisoindoline **218** (5.0 g, 17.91 mmol, 1.0 equiv.) and palladium (476 mg, 448 μmol, 0.025 equiv., 10 % on carbon) in glacial

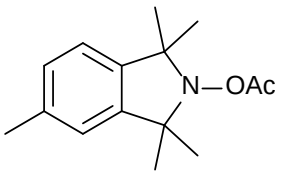
acetic acid (120 mL) was shaken under hydrogen gas (50 psi) for 4 h. The reaction mixture was then filtered through Celite and concentrated *in vacuo*. The resulting residue was basified with NaOH (5 M, pH ≥12) and extracted with Et_2O (60 mL x 5). The combined Et_2O extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to afford white crystals of 1,1,3,3,5-pentamethylisoindoline **219** (3.26 g, 96 %), which was used in subsequent reactions without further purification. Mp. 52 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.43 (s, 6 H, CH_3),1.44 (s, 6 H, CH_3), 1.78 (s, NH) 2.37 (s, 3 H, CH_3), 6.93 (s, 1 H, Ar-H), 7.01 (d, J = 7.6 Hz, 1 H, Ar-H), 7.06 (d, J = 7.6 Hz, 1 H, Ar-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.3 (Ar-*C*H_3), 31.9 (C*C*H_3), 32.0 (C*C*H_3), 62.5 (*C*CH_3), 62.6 (*C*CH_3), 121.2 (Ar-*C*), 122.0 (Ar-*C*), 128.0 (Ar-*C*), 136.8 (Ar-*C*), 146.0 (Ar-*C*), 149.0 (Ar-*C*) ppm. HRMS (ES): *m*/*z* (%) = 190 (25) $[M + H]^+$; calcd. for $C_{13}H_{19}N$ $[M + H]^+$ 190.1596; found 190.1590.

3.5.20. Synthesis of 1,1,3,3,5-Pentamethylisoindol-2-yloxyl 220



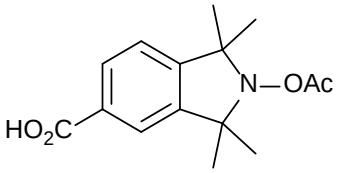
m-Chloroperoxybenzoic acid (5.02 g, 17.21 mmol, 1.2 equiv., 77 %) was added to an ice-cooled solution of 1,1,3,3,5-pentamethylisoindoline **219** (2.72 g, 14.35 mmol, 1.0 equiv.) in DCM (80 mL). The resulting solution was stirred for 1 h at 0 °C and then at RT for a further 2 h. Excess DCM was added to dissolve the precipitate that formed during the reaction. The bright-yellow organic phase was washed successively with aqueous HCl (0.5 M, 40 mL), NaOH (2 M, 40 mL x 3) and brine (50 mL) solutions, then dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated *in vacuo* to give 1,1,3,3,5-pentamethylisoindol-2-yloxyl **220** as a bright-yellow solid (2.78 g, 95 %). Mp. 90 °C. HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 227 (40) $[M + Na]^+$; calcd. for $C_{13}H_{18}NNaO$ $[M + Na]^+$ 227.1286; found 227.1290.

3.5.21. Synthesis of N-Acetoxy-1,1,3,3,5-pentamethyl isoindoline 221



A mixture of 1,1,3,3,5-pentamethylisoindol-2-yloxyl **220** (1.0 g, 4.90 mmol, 1 equiv.) and palladium (130 mg, 122 μmol, 0.025 equiv., 10 % on carbon) in dry THF (25 mL) was stirred under a balloon of hydrogen gas for 20 min. The reaction mixture was then cooled to 0 °C and Et_3N (1.37 mL, 9.79 mmol, 2.0 equiv.) and AcCl (869 μL, 12.24 mmol, 2.5 equiv.) were added dropwise. The resulting solution was stirred at 0 °C for 30 min and then at RT for a further 1 h. The mixture was then filtered through Celite and concentrated *in vacuo*. The residue was dissolved in EtOAc (150 mL) and washed with brine (40 mL). The organic phase was dried with anhydrous Na_2SO_4 and filtered. The filtrate was concentrated *in vacuo* to give *N*-acetoxy-1,1,3,3,5-pentamethylisoindoline **221** as a white solid (1.10 g, 91 %). Mp. 113 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.38 (br. s, 6 H, 2 CH_3), 1.47 (br. s, 6 H, 2 CH_3), 2.21 (s, 3 H, CH_3), 2.39 (s, 3 H, CH_3), 6.93 (s, 1 H, Ar-H), 7.00 (d, J = 8.0 Hz, 1 H, Ar-H), 7.07 (d, J = 8.0 Hz, 1 H, Ar-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.3, 21.5, 25.4, 28.9, 68.0, 68.2, 121.3, 122.1, 128.5, 137.4, 141.0, 143.9, 171.9 ppm. HRMS (ES): *m*/*z* (%) = 248 (95) $[M + H]^+$; calcd. for $C_{15}H_{23}NO_2$ $[M + H]^+$ 248.1651; found 248.1645. FTIR (ATR): $\tilde{\nu}_{max}$ = 1758 (s, C=O) cm^{-1}.

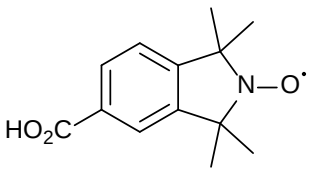
3.5.22. Synthesis of 2-Acetoxy-1,1,3,3-tetramethylisoindoline-5-carboxylic Acid 222



A solution containing *N*-acetoxy-1,1,3,3,5-pentamethylisoindoline **221** (690 mg, 2.79 mmol, 1.0 equiv.), $MgSO_4$ (169 mg, 1.39 mmol, 0.5 equiv.) and a freshly prepared aqueous $KMnO_4$ solution (27.6 mL, 11.16 mmol, 4.0 equiv., 0.4 M) was stirred at 70 °C in *t*BuOH (15 mL) for 1 d. The reaction mixture was cooled to RT, treated with *i*PrOH (10 mL) and stirred overnight. The mixture was filtered through Celite and

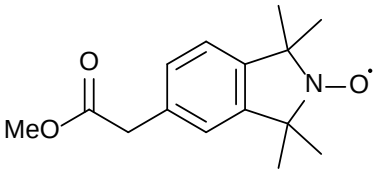
the solvent removed *in vacuo*. The resulting residue was dissolved in EtOAc (200 mL), washed brine (50 nL) and dried over anhydrous Na_2SO_4. The solution was filtered and the filtrate concentrated *in vacuo*. Purification of the resulting residue by silica gel flash chromatography (eluent: $CHCl_3$ to $CHCl_3$/EtOAc, 9:1) afforded 2-acetoxy-1,1,3,3-tetramethylisoindoline-5-carboxylic Acid **222** as a pale-yellow solid (498 mg, 65 %). Mp.129 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.45 (br. s, 6 H, 2 CH_3), 1.54 (br. s, 6 H, 2 CH_3), 2.23 (s, 3 H, CH_3), 7.25 (d, J = 7.6 Hz, 1 H, Ar-H), 7.91 (s, 1 H, Ar-H), 8.06 (d, J = 7.6 Hz, 1 H, Ar-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.3 (C=O*C*H_3), 25.2 (C*C*H_3), 25.3 (C*C*H_3), 28.7 (C*C*H_3), 28.9 (C*C*H_3), 68.2 (*C*CH_3), 68.4 (*C*CH_3), 121.9 (Ar-*C*), 123.8 (Ar-*C*), 129.4 (Ar-*C*), 130.4 (Ar-*C*), 144.6 (Ar-*C*), 150.2 (Ar-*C*), 171.5 (C=O), 171.7 (C=O) ppm. HRMS (ES): *m*/*z* (%) = 278 (89) $[M + H]^+$; calcd. for $C_{15}H_{20}NO_4$ $[M + H]^+$ 278.1392; found 278.1383. FTIR (ATR): $\tilde{\nu}_{max}$ = 3400–2800 (br, m, O–H), 1771 (s, C=O), 1686 (s, C=O) cm^{-1}.

3.5.23. Synthesis of CTMIO 70 from 2-Acetoxy-1,1,3,3-tetramethylisoindoline-5-carboxylic Acid 222



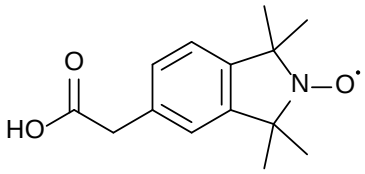
2-Acetoxy-1,1,3,3-tetramethylisoindoline-5-carboxylic acid **222** (100 mg, 360 μmol) was stirred in a solution of NaOH (3 mL, 1 M) and EtOH (1 mL) at 0 °C for 30 min. The cooling bath was removed and the reaction was stirred at RT overnight. The aqueous layer was diluted with H_2O (25 mL) and washed with Et_2O (10 mL) and then acidified to pH 1 with HCl (2 M). The acidic aqueous layer was extracted with Et_2O (4 × 30 mL) and the combined organic extracts washed with brine (30 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated *in vacuo* and recrystallized from MeCN to give CTMIO **70** as a yellow solid (82 mg, 97 %). Mp. 211 °C (Lit.,[14] 214–218 °C).

3.5.24. Synthesis of 5-Methoxycarbonylmethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 224



m-Chloroperoxybenzoic acid (471 mg, 1.62 mmol, 1.5 equiv., 77%) was added to a solution of methyl 2-methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindoline **223** (300 mg, 1.08 mmol, 1 equiv.) in DCM (150 mL) at 0 ^{o}C. The reaction mixture was stirred for 30 min and then at RT for further 3.5 h. The resulting solution was washed with saturated $NaHCO_3$ (30 mL x 2) and brine solutions and dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude residue purified by flash column chromatography (Hexane/EtOAc, 4:1) to give 5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **224** as bright yellow solid (253 mg, 89%). Mp. 96-96.5 °C. HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 263.1500 (100) $[M + H]^+$, 264.1555 (30) $[M + 2H]^+$; calcd. for $C_{15}H_{20}NO_3^{\bullet}$: 262.1443; found 262.1426. ATR-FTIR: υ_{max}= 2976 (m, Ar C-H), 1737 (s, C=O), 1155 (s, C-O) cm^{-1}.

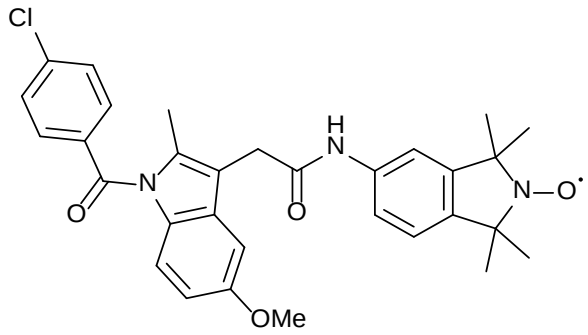
3.5.25. Synthesis of 5-Carboxymethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 205



NaOH (4 mL, 2 M) was added to a solution of 5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **224** (250 mg, 953 μmol, 1 equiv.) in MeOH (6 mL) and the resulting reaction mixture was stirred for 2 h at 60 ^{o}C. The reaction mixture was cooled to RT and diluted with H_2O (30 mL). The aqueous layer was washed with Et_2O (30 mL) and acidified (pH 1) with HCl (2 M). The aqueous layer was extracted with Et_2O (60 mL x 3) and the combined organic extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give 5-carboxymethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **205** as yellow solid (222 mg, 94%). Mp. 123-124 °C. HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 249.1320 (50) $[M + H]^+$; calcd. for $C_{14}H_{18}NO_3^{\bullet}$: 248.1287; found 248.1288. ATR-

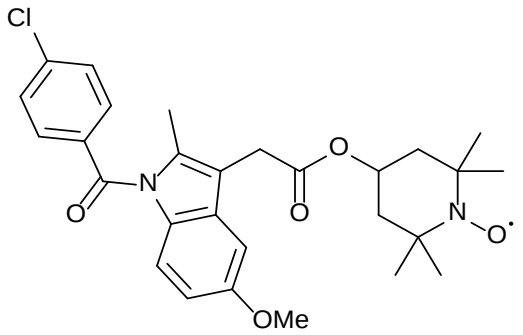
FTIR: υ_{max}= 3000 (s, br, O-H), 2977 (m, Ar C-H), 1729 (s, C=O), 1144 (s, C-O) cm^{-1}.

3.5.26. Synthesis of 2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)acetamide 143



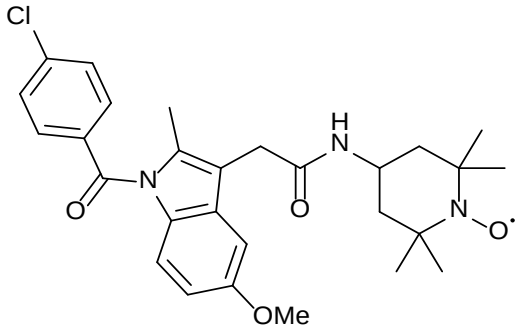
A solution of indomethacin **163** (162 mg, 453 µmol, 1 equiv.), 5-amino-1,1,3,3-tetramethylisoindolin-2-yloxyl **108** (102.24 mg, 498 µmol, 1.1 equiv.), EDC (95.5 mg, 498 µmol, 1.1 equiv.), and DMAP (11 mg, 90.6 µmol, 0.2 equiv.) in DCM (10 mL) was stirred under Ar overnight. The resulting reaction mixture was diluted (DCM, 150 mL), washed with HCl (1 M, 30 mL x 2) and brine (40 mL), then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 3:1) and recrystallized from cyclohexane/EtOAc give 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(1,1,3,3-tetramethylisoindolin -2-yloxyl-5-yl)acetamide **143** as light yellow crystals (228 mg, 92%). Mp. 164-166 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.49 (br, s, 3 H, C*H*$_3$), 3.83 (br, s, 3 H, OC*H*$_3$), 3.88 (br, s, 2 H, C*H*$_2$), 6.74 (br, d, 1 H, *J* = 8.4 Hz, Ar-*H*), 6.9 (br, d, 1 H, *J* = 8.4 Hz, Ar-*H*), 6.96 (br, s, 1 H, Ar-*H*), 7.51 (br, d, 2 H, *J* = 6 Hz, Ar-*H*), 7.7 (br, d, 2 H, *J* = 6 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 567.1942 (100) $[M + Na]^+$; calcd. for $C_{31}H_{31}ClN_3NaO_4^{\bullet}$: 567.1901; found 567.1942; calcd. for $C_{31}H_{31}ClN_3O_4^{\bullet}$: 544.2003; found 544.2010. ATR-FTIR: υ_{max}= 3299 (w, N-H), 2973 (m, Ar C-H), 1677 (s, C=O), 1599 (C=O), 1313 (C-N), 1223 (C-O) cm^{-1}.

3.5.27. Synthesis of 2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2,2,6,6-tetramethylpiperidin-1-yloxyl-yl)acetate 226



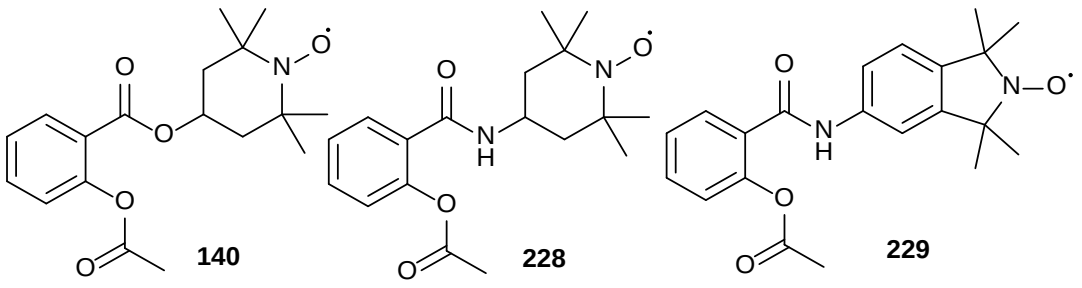
A solution of indomethacin **163** (200 mg, 559 μmol, 1 equiv.), TEMPOL **102** (106 mg, 615 μmol, 1.1 equiv.), EDC (118 mg, 615 μmol, 1.1 equiv.), and DMAP (14 mg, 112 μmol, 0.2 equiv.) in DCM (10 mL) was stirred under Ar overnight. The resulting reaction mixture was diluted (DCM, 150 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 3:1) to give 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(2,2,6,6-tetramethylpiperidin-1-yloxyl-yl) acetate **226** as a pale orange solid (278 mg, 97%). Mp. 71-72 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.44 (br, s, 3 H, C*H*$_3$), 3.70 (br, s, 2 H, C*H*$_2$), 3.86 (br, s, 3 H, OC*H*$_3$), 6.69 (br, d, 1 H, *J* = 7.8 Hz, Ar-*H*), 6.87 (br, d, 1 H, *J* = 8.4 Hz, Ar-*H*), 6.99 (br, s, 1 H, Ar-*H*), 7.49 (br, d, 2 H, *J* = 6 Hz, Ar-*H*), 7.67 (br, d, 2 H, *J* = 6.6 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): *δ* = 21.0 (*C*H$_3$), 30.9 (*C*H$_2$), 52.2 (O*C*H$_3$), 117.3 (Ar-*C*), 117.31 (Ar-*C*), 120.0 (Ar-*C*), 123.3 (Ar-*C*), 124.4 (Ar-*C*), 142.5 (Ar-*C*), 144.3 (Ar-*C*), 165.1 (Ar-*C*), 170.4 (*C*=O), 207.0 (*C*=O). HRMS (ES): *m*/*z* (%) = 512.1929 (100) $[M + H]^+$; calcd. for $C_{28}H_{32}ClN_2O_5^{\bullet}$: 511.2000; found 511.1925. ATR-FTIR: υ_{max}= 2973 (m, Ar C-H), 1732 (s, C=O), 1680 (C=O), 1314 (C-N), 1141 (C-O) cm^{-1}.

3.5.28. Synthesis of 2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2,2,6,6-tetramethylpiperidin-1-yloxyl-4-yl)acetamide 227



A solution of indomethacin **163** (209 mg, 539 μmol, 1 equiv.), 5-amino-TEMPO **76** (110 mg, 642 μmol, 1.1 equiv.), EDC (123 mg, 642 μmol, 1.1 equiv.), and DMAP (14.3 mg, 117 μmol, 0.2 equiv.) in DCM (10 mL) was stirred under Ar overnight. The resulting reaction mixture was diluted (DCM, 150 mL), washed with HCl (1 M, 30 mL x 2) and brine (40 mL), then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 3:1) and recrystallized from MeOH give reddish brown crystals of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(2,2,6,6-tetramethylpiperidin-1-yloxyl-4-yl)acetamide **227** (259 mg, 87%). Mp. 199-201 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.46 (br, s, 3 H, C*H*$_3$), 3.7 (br, s, 2 H, C*H*$_2$), 3.91 (br, s, 3 H, OC*H*$_3$), 5.28 (br, s, 2 H, Ar-*H*), 6.69 (br, d, 2 H, Ar-*H*), 7.28 (br, s, 2 H, Ar-), 7.6 (br, d, 2 H, Ar-). HRMS (ES): *m*/*z* (%) = 511.5199 (10) $[M + H]^+$, 522.2062 (50) $[M + Na]^+$; calcd. for $C_{28}H_{33}ClN_3O_4^{\bullet}$: 510.2160; found 510.2158. ATR-FTIR: υ_{max}= 1079 (w, N-H), 2973 (m, Ar C-H), 1636 (s, C=O), 1555 (C=O), 1350 (C-N), 1111 (C-O) cm^{-1}.

3.5.29. General Procedure for the S*ynthesis of Salicylic Acid Derivatives 140, 228 and 229*



140 **228** **229**

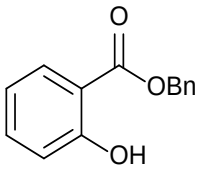
A solution of aspirin **136** (150 mg, 833 μmol, 1 equiv.), appropriate nitroxide **76**, **102** or **108** (1 mmol, 1.2 equiv.), EDC (191.5 mg, 1 mmol, 1.2 equiv.), and DMAP (13 mg, 104 μmol, 0.125 equiv.) in DCM (10 mL) was stirred under Ar for 1 d. The resulting reaction mixture was diluted (DCM, 150 mL), washed HCl (2 M, 30 mL) and brine (30 mL) solutions, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography ($CHCl_3$) and then recrystallization from cyclohexane/EtOAc (except **228**) to give the corresponding salicylate-nitroxide.

Compound **140**: Reddish brown solid (251 mg, 90%). Mp. 52-53 ^oC. HPLC purity (>95%). HRMS (ES): *m/z* (%) = 335.1688 (10) $[M + H]^+$; calcd. for $C_{18}H_{24}NO_5^{\bullet}$: 334.1654; found 334.1654. ATR-FTIR: υ_{max}= 2977 (w, ArC-H), 1724 (s, C=O), 1255 (s, C-O) cm^{-1}.

Compound **228**: Reddish brown solid (153 mg, 55%). Mp. 50-52 ^oC. HPLC purity (>95%). HRMS (ES): *m/z* (%) = 334.1850 (25) $[M + H]^+$; calcd. for $C_{18}H_{25}N_2O_4^{\bullet}$: 333.1814; found 333.1817. ATR-FTIR: υ_{max}= 3314 (m, N-H), 2976 (w, ArC-H), 1640 (s, C=O), 1546 (s, C=O), 1229 (s, C-O) cm^{-1}.

Compound **229**: Yellow solid (162 mg, 53%). Mp. 101-102 ^oC. HPLC purity (>95%). HRMS (ES): *m/z* (%) = 168.1689 (20) $[M + H]^+$; calcd. for $C_{21}H_{23}N_2O_4^{\bullet}$: 367.1658; found 367.1660. ATR-FTIR: υ_{max}= 3340 (m, N-H), 2971 (w, ArC-H), 1681 (s, C=O), 1543 (s, C=O), 1255 (s, C-O) cm^{-1}.

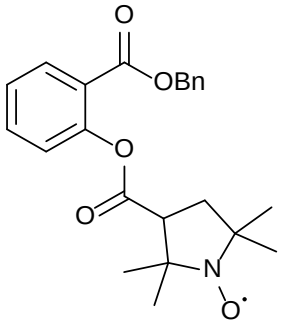
3.5.30. Synthesis of Benzyl 2-hydroxybenzoate 231



$NaHCO_3$ (1.46 g, 17.4 mmol, 1.2 equiv.) was added to a solution salicylic acid **135** (2 g, 14.5 mmol, 1 equiv.) in DMF (20 mL) and the resulting mixture was stirred at 70 ^oC for 10 min. The temperature was reduced to 50 ^oC followed by the addition of benzylbromide (1.81 mL, 15.2 mmol, 1.05 equiv.). Then reaction mixture was stirred

for 4 h and then allowed to cool to RT. H_2O (50 mL) was added and the crude product was extracted with EtOAc (60 mL x 4). The combined EtOAc extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. Purification of the crude residue by silica gel chromatography (Hexane/EtOAc, 5:0.2) afforded compound **231** as clear oil (3.11 g, 94%). HPLC purity (>99%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.42 (s, 2 H, C*H*$_2$), 6.9 (m, 1 H, Ar-*H*), 7.4 (m, 6 H, Ar-*H*), 7.91 (q, 1 H, Ar-*H*), 10.78 (s, 1 H, O-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 66.98 (*C*H$_2$), 112.4 (Ar-*C*), 117.6 (Ar-*C*), 119.2 (Ar-*C*), 128.3 (Ar-*C*), 128.6 (Ar-*C*), 128.7 (Ar-*C*), 130.0 (Ar-*C*), 135.3 (Ar-*C*), 135.8 (Ar-*C*), 161.8 (Ar-*C*), 170.0 (*C*=O). ATR-FTIR: υ_{max}= 3188 (m, br, O-H), 3000 (m, Ar C-H), 1670 (s, C=O), 1086 and 1133 (s, C-O) cm^{-1}.[28]

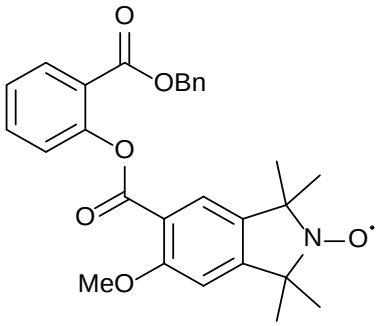
3.5.31. Synthesis of 2-(Benzyloxy)carbonyl)phenyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-carboxylate 232



A solution of benzyl salicylate **231** (135 mg, 591 µmol, 1.1 equiv.), carboxy-PROXYL **204** (100 mg, 537 µmol, 1 equiv.), EDC (113 mg, 591 µmol, 1.1 equiv.), and DMAP (14.4 mg, 118 µmol, 0.2 equiv.) in DCM (10 mL) was stirred under Ar for 1 day. The resulting reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 5:1) to give 2-(benzyloxycarbonyl)phenyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-carboxylate **232** as yellow oil (183 mg, 78%). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 5.33 (s, br, 2 H, C*H*$_2$), 6.9 (s, br, 1 H, Ar-*H*), 7.4 (br, m, 7 H, Ar-*H*), 7.62 (s, br, 1 H, Ar-*H*), 8.1 (d, br, 1 H, *J* = 7.6 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 419.1699 (100) $[M + Na]^+$; calcd. for $C_{23}H_{26}NO_5^{\bullet}$: 396.1811; found 396.1809.

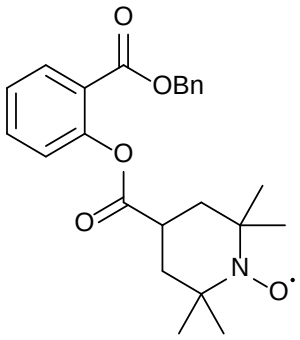
ATR-FTIR: υ_{max}= 2975 (w, ArC-H), 1761 (s, C=O), 1719 (s, C=O), 1251 (s, C-N), 1134 and 1075 (s, C-O) cm^{-1}.

3.5.32. Synthesis of 2-(Benzyloxycarbonyl)phenyl-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carboxylate 235



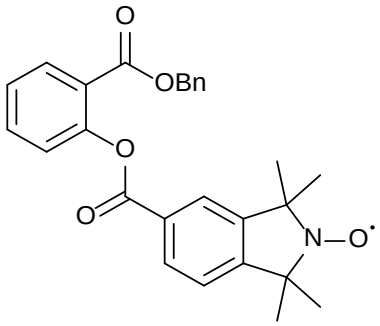
A solution of benzyl salicylate **231** (60 mg, 262 µmol, 1.1 equiv.), 5-carboxy-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **175** (63 mg, 238.4 µmol, 1 equiv.), EDC (50.3 mg, 262 µmol, 1.1 equiv.), and DMAP (6 mg, 48 µmol, 0.2 equiv.) in DCM (10 mL) was stirred under Ar for 1 day. The resulting reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 5:1) and then recrystallization from cyclohexane/EtOAc to give 2-(benzyloxycarbonyl)phenyl-6-methoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carboxylate **235** as a yellow solid (82 mg, 73%). Mp. 142-143 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 4.21 (br, s, 3 H, OCH_3), 5.27 (s, br, 2 H, CH_2), 7.27 (s, br, 2 H, Ar-*H*), 7.4 (br, m, 2 H, Ar-*H*), 7.62 (s, br, 1 H, Ar-*H*), 8.11 (d, br, 1 H, *J* = 7.8 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 497.1806 (40) $[M + Na]^+$; calcd. for $C_{28}H_{28}NO_6^{\bullet}$: 474.1917; found 474.1920. ATR-FTIR: υ_{max}= 2979 (w, ArC-H), 1748 (s, C=O), 1718 (s, C=O), 1294 (s, C-N), 1192 (s, C-O) cm^{-1}.

3.5.33. Synthesis of 2-(Benzyloxycarbonyl)phenyl-2,2,6,6-tetramethylpiperidin-1-yloxyl-4-carboxylate 233



A solution of benzyl salicylate **231** (63 mg, 275 μmol, 1.1 equiv.), carboxy-TEMPO **8** (50 mg, 250 μmol, 1 equiv.), EDC (53 mg, 275 μmol, 1.1 equiv.), and DMAP (6 mg, 50 μmol, 0.2 equiv.) in DCM (8 mL) was stirred under Ar for 1 day. The resulting reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 5:1) and then recrystallization from cyclohexane to give 2-(benzyloxycarbonyl)phenyl-2,2,6,6-tetramethylpiperidin-1-yloxyl-4-carboxylate **233** as a light reddish-brown fluffy solid (73 mg, 71%). Mp. 112-113 °C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 5.34 (s, br, 2 H, CH_2), 7.1 (d, br, 1 H, Ar-*H*), 7.34 (br, m, 7 H, Ar-*H*), 7.6 (s, br, 1 H, Ar-*H*), 8.1 (d, br, 1 H, *J* = 7.2 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 412.2126 (30) $[M + 2H]^+$, 433.1872 $[M + Na]^+$, 449.2757 $[M + K]^+$; calcd. for $C_{24}H_{28}NO_5^{\bullet}$: 410.1967; found 410.1967. ATR-FTIR: υ_{max}= 2981 (w, ArC-H), 1745 (s, C=O), 1722 (s, C=O), 1257 (s, C-N), 1085 (s, C-O) cm^{-1}.

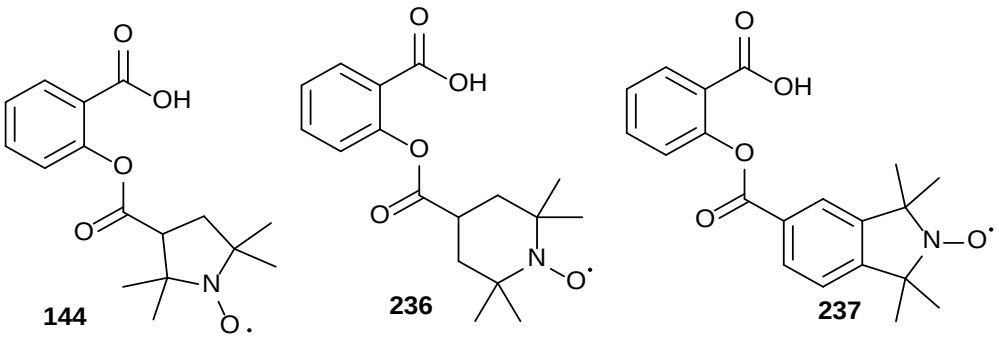
3.5.34. Synthesis of 2-(Benzyloxycarbonyl)phenyl-1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carboxylate 234



A solution of benzyl salicylate **231** (70 mg, 305.4 μmol, 1.1 equiv.), CTMIO **70** (65 mg, 278 μmol, 1 equiv.), EDC (59 mg, 305.4 μmol, 1.1 equiv.), and DMAP (7 mg, 56 μmol, 0.2 equiv.) in DCM (8 mL) was stirred under Ar for 1 day. The resulting

reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 5:1) and then recrystallization from cyclohexane/EtOAc to give 2-(benzyloxycarbonyl)phenyl-1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carboxylate **234** as a yellow crystalline solid (90 mg, 72%). Mp. 97-98 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 5.25 (s, br, 2 H, C*H*$_2$), 7.26 (s, br, 7 H, Ar-*H*), 7.42 (br, s, 1 H, Ar-*H*), 7.66 (s, br, 1 H, Ar-*H*), 8.17 (d, br, 1 H, *J* = 7.2 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 446.1880 (20) $[M + 2H]^+$; calcd. for $C_{27}H_{26}NO_5^{\bullet}$: 444.1811; found 444.1813. ATR-FTIR: υ_{max}= 3038 (w, ArC-H), 1744 (s, C=O), 1705 (s, C=O), 1290 (s, C-N), 1192 (s, C-O) cm^{-1}.

3.5.35. General Procedure for the Synthesis of Carboxylic Acid Nitroxides (144, 236 and 237) via Debenzylation of Benzyl Benzoates 232-234



A solution of benzyl benzoate nitroxide **232-234** (377 µmol, 1 equiv.) in MeOH (10 mL) was hydrogenated at 30 psi over 10% Pd/C for 4 h in a Parr Hydrogenator. The solution was filtered through Celite and the filtrate stirred over PbO_2 (94.25 µmol, 0.25 equiv.) for 20 min. The solution was filtered and concentrated under reduced pressure. The crude residue was purified by purified by silica gel column chromatography (EtOAc and 0.2% AcOH) and then recrystallization from H_2O/MeOH to give the corresponding carboxylic acid nitroxide (**144**, **236** and **237**).

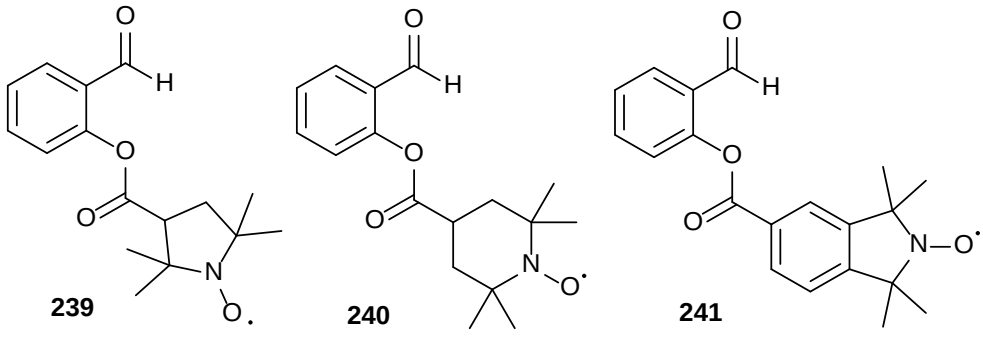
2-((2,2,5,5-Tetramethylpyrrolidin-1-yloxyl-3-carbonyl)oxy)benzoic acid **144** from **232**: yellow crystalline solid (57 mg, 49%). Mp. 161-162 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.18 (s, br, 1 H, Ar-*H*), 7.4 (br, s, 1 H, Ar-*H*), 7.66 (s, br, 1 H, Ar-*H*), 8.1 (d, br, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) =

307.1377 (15) $[M + H]^+$; calcd. for $C_{16}H_{20}NO_5^{\bullet}$: 306.1341; found 306.1341. ATR-FTIR: υ_{max}= 3500-2400 (m, br, O-H), 2976 (w, ArC-H), 1766 (s, C=O), 1713 (s, C=O), 1204 (s, C-N), 1126 (s, C-O) cm^{-1}.

2-((2,2,6,6-Tetramethylpiperidin-1-yloxyl-4-carbonyl)oxy)benzoic acid **236** from **233**: light reddish-brown solid (56 mg, 46%). Mp. 149 ^oC (dec.). HPLC purity (>95%). ^1H NMR ($CDCl_3$, 600 MHz): δ = 7.17 (s, br, 1 H, Ar-*H*), 7.4 (s, br, 1 H, Ar-*H*), 7.67 (s, br, 1 H, Ar-*H*), 8.1 (s, br, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 321.1532 (100) $[M + H]^+$; calcd. for $C_{17}H_{22}NO_5^{\bullet}$: 320.1498; found 320.1499. ATR-FTIR: υ_{max}= 3500-2400 (m, br, O-H), 1755 (s, C=O), 1716 (s, C=O), 1241 (s, C-N), 1145 (s, C-O) cm^{-1}.

2-((1,1,3,3-Tetramethylisoindolin-2yloxyl-5-carbonyl)oxy)benzoic acid **237** from **234**: yellow crystalline solid (55 mg, 41%). Mp. 193 ^oC (dec.). HPLC purity (>95%).^1H NMR ($CDCl_3$, 600 MHz): δ = 7.27 (s, br, 1 H, Ar-*H*), 7.41 (s, br, 1 H, Ar-*H*), 7.68 (s, br, 1 H, Ar-*H*), 8.12 (s, br, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 356.1409 (30) $[M + 2H]^+$; calcd. for $C_{20}H_{20}NO_5^{\bullet}$: 354.1341; found 354.1341. ATR-FTIR: υ_{max}= 3350-2400 (m, br, O-H), 1734 (s, C=O), 1688 (s, C=O), 1288 (s, C-N), 1196 (s, C-O) cm^{-1}.

3.5.36. General Procedure for the Synthesis of Formyl-Nitroxides 239-241



A solution of salicylaldehyde **238** (1.31 mmol, 1.05 equiv.), appropriate carboxy-nitroxide **8**, **70** and **204** (1.25 mmol, 1 equiv.), EDC (1.37 mmol, 1.1 equiv.), and DMAP (156 μmol, 0.125 equiv.) in DCM (10 mL) was stirred under Ar overnight. The resulting reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude

residue was purified by silica gel column chromatography ($CHCl_3$ or Hexane/EtOAc, 3:1) and then recrystallization from cyclohexane/EtOAc to give the corresponding formyl-o-nitroxides (**239-241**).

2-Formylphenyl 2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-carboxylate **239**: yellow crystalline solid (319 mg, 88%). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.19 (s, br, 1 H, Ar-*H*), 7.47 (d, br, 1 H, Ar-*H*), 7.7 (s, br, 1 H, Ar-*H*), 7.9 (s, br, 1 H, Ar-*H*), 10.1 (s, br, *H*C=O). HRMS (ES): *m*/*z* (%) = 291.1428 (30) $[M + H]^+$; calcd. for $C_{16}H_{20}NO_4^{\bullet}$: 290.1392; found 290.1395. ATR-FTIR: υ_{max}= 3350-2400 (m, br, O-H), 1734 (s, C=O), 1688 (s, C=O), 1288 (s, C-N), 1196 (s, C-O) cm^{-1}.

2-Formylphenyl 2,2,6,6-tetramethylpiperidin-1-yloxyl-4-carboxylate **240**: light reddish-brown, fluffy crystals (327 mg, 86%). Mp. 94-95 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.18 (s, br, 1 H, Ar-*H*), 7.47 (d, br, 1 H, Ar-*H*), 7.69 (s, br, 1 H, Ar-*H*), 7.91 (d, br, 1 H, *J* = 7.2 Hz, Ar-*H*), 10.1 (s, br, *H*C=O). HRMS (ES): *m*/*z* (%) = 327.1442 (100) $[M + Na]^+$; calcd. for $C_{17}H_{22}NO_4^{\bullet}$: 304.1549; found 304.1550. ATR-FTIR: υ_{max}= 3079 (m, ArC-H), 1745 (s, C=O), 1699 (s, C=O), 1230 (s, C-N), 1154 (s, C-O) cm^{-1}.

2-Formylphenyl 1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carboxylate **241**: yellow crystalline solid (372 mg, 88%). Mp. 151-152 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.34 (d, br, 1 H, *J* = 7.2 Hz, Ar-*H*), 7.49 (t, br, 1 H, *J* = 7.2 Hz, Ar-*H*), 7.72 (t, br, 1 H, Ar-*H*), 7.98 (d, br, 1 H, *J* = 7.2 Hz, Ar-*H*), 10.21 (s, br, *H*C=O). HRMS (ES): *m*/*z* (%) = 361.1292 (50) $[M + Na]^+$; calcd. for $C_{20}H_{20}NO_4^{\bullet}$: 338.1392; found 338.1391. ATR-FTIR: υ_{max}= 2978 (m, ArC-H), 1749 (s, C=O), 1708 (s, C=O), 1234 (s, C-N), 1197 (s, C-O) cm^{-1}.

3.5.37. General Procedure for the Oxidation of Formyl Nitroxides 239-241 to the Corresponding Carboxylic Acids 144, 236 and 237

Method A

KH_2PO_4 (48.3 mg, 355 µmol, 2 equiv. in 0.5 mL H_2O) and H_2O_2 (30 µL, 355 µmol, 1.5 equiv. 30% in H_2O) were added to a solution of appropriate formyl nitroxide **239-241** (177.3 µmol, 1 equiv.) in MeCN (5 mL) at 0 ^{o}C. $NaClO_2$ (40.3 mg, 356

µmol, 2 equiv. in 0.5 mL H_2O) was then added dropwise and the resulting solution stirred for 2 h. The reaction mixture was diluted with H_2O and the aqueous layer extracted with DCM (30 mL x 3). The DCM extract was washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/0.01%AcOH) and then recrystallization from H_2O/MeOH to give the corresponding carboxylic acid nitroxide (**144**, **236** and **237**).

2-((2,2,5,5-Tetramethylpyrrolidin-1-yloxyl-3-carbonyl)oxy)benzoic acid **144** from **239**: yellow crystalline solid (319 mg, 88%). Mp. 161-162 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.18 (s, br, 1 H, Ar-*H*), 7.7.4 (br, s, 1 H, Ar-*H*), 7.66 (s, br, 1 H, Ar-*H*), 8.1 (d, br, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 307.1377 (15) $[M + H]^+$; calcd. for $C_{16}H_{20}NO_5^{\bullet}$: 306.1341; found 306.1341. ATR-FTIR: υ_{max}= 3500-2400 (m, br, O-H), 2976 (w, ArC-H), 1766 (s, C=O), 1713 (s, C=O), 1204 (s, C-N), 1126 (s, C-O) cm^{-1}.

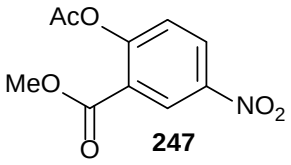
2-((2,2,6,6-Tetramethylpiperidin-1-yloxyl-4-carbonyl)oxy)benzoic acid **236** from **240**: light reddish-brown, fluffy crystals (327 mg, 86%). Mp. 149 ^{o}C (dec.). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.17 (s, br, 1 H, Ar-*H*), 7.4 (s, br, 1 H, Ar-*H*), 7.67 (s, br, 1 H, Ar-*H*), 8.1 (s, br, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 321.1532 (100) $[M + H]^+$; calcd. for $C_{17}H_{22}NO_5^{\bullet}$: 320.1498; found 320.1499. ATR-FTIR: υ_{max}= 3500-2400 (m, br, O-H), 1755 (s, C=O), 1716 (s, C=O), 1241 (s, C-N), 1145 (s, C-O) cm^{-1}.

2-((1,3,3-Tetramethylisoindolin-2-yloxyl-5-carbonyl)oxy)benzoic acid **237** from **241**: yellow crystalline solid (372 mg, 88%). Mp. 193 ^{o}C (dec.). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.27 (s, br, 1 H, Ar-*H*), 7.41 (s, br, 1 H, Ar-*H*), 7.68 (s, br, 1 H, Ar-*H*), 8.12 (s, br, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 356.1409 (30) $[M + 2H]^+$; calcd. for $C_{20}H_{20}NO_5^{\bullet}$: 354.1341; found 354.1341. ATR-FTIR: υ_{max}= 3350-2400 (m, br, O-H), 1734 (s, C=O), 1688 (s, C=O), 1288 (s, C-N), 1196 (s, C-O) cm^{-1}.

Method B

$KMnO_4$ (56 mg, 355 µmol, 2 equiv.) was added to a solution of appropriate formyl nitroxide **239-241** (177.3 µmol, 1 equiv.) in acetone (5 mL) at 0 °C under Ar. The reaction mixture was stirred for 2 h, followed by the addition of dilute H_2SO_4 (0.5 M). The aqueous layer was extracted with DCM and the combined DCM extracts washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was recrystallization from H_2O/MeOH to give the corresponding carboxylic acid nitroxide (**144** (86%), **236** (85%) and **237** (85%).

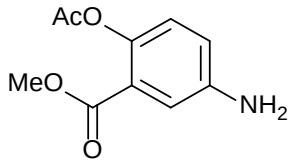
3.5.38. Synthesis of Methyl 2-acetoxy-5-nitrobenzoate 233



247

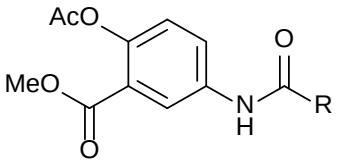
A suspension of methyl 2-hydroxy-5-nitrobenzoate **246** (650 mg, 3.3 mmol, 1 equiv.) in THF (15 mL) under Ar was cooled to 0 °C. TEA (1.15 mL, 8.24 mmol, 2.5 equiv.) and then AcCl (459 µL, 6.59 mmol, 2 equiv.) were added dropwise and the resulting mixture stirred for 2 h. The reaction mixture was diluted with H_2O and the aqueous layer extracted with DCM (60 mL x 4). The combined DCM extracts were washed brine (50 mL) and dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography ($CHCl_3$/Hexane, 1:1) and then recrystallization from cyclohexane to give methyl 2-acetoxy-5-nitrobenzoate **247** as a white crystalline solid (91%). Mp. 75-76 °C (Lit.,[37] 73-74 °C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.4 (s, 3 H, C=OCH_3), 3.94 (s, 3 H, OCH_3), 7.3 (d, 1 H, *J* = 8.4 Hz, Ar-*H*), 8.42 (dd, 1 H, *J* = 8.4 Hz, 2.8 Hz, Ar-*H*), 8.9 (d, 1 H, *J* = 2.8 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 20.9 (C=O*C*H$_3$), 52.8 (O*C*H$_3$), 124.4 (Ar-*C*), 125.3 (Ar-*C*), 127.4 (Ar-*C*), 128.5 (Ar-*C*), 145.3 (Ar-*C*), 155.3 (Ar-*C*), 163.0 (*C*=O), 168.8 (*C*=O). ATR-FTIR: υ_{max}= 3102 (w, Ar C-H), 1759 (s, C=O), 1727 (s, C=O), 1529 (s, NO_2), 1190 (s, C-O) cm^{-1}.

3.5.39. Synthesis of Methyl 2-acetoxy-5-aminobenzoate 248



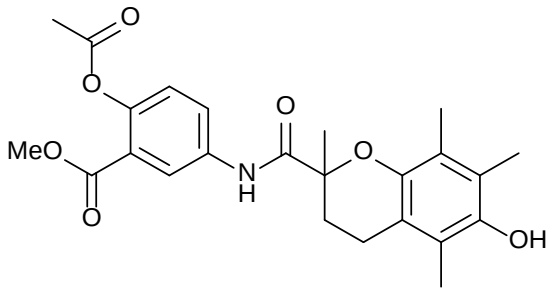
A solution of methyl 2-acetoxy-5-nitrobenzoate **247** (500 mg, 2.09 mmol, 1 equiv.) in EtOAc (20 mL) was hydrogenated at 50 psi over 10% Pd/C (50 mg) for 4 h in a Parr Hydrogenator. The resulting solution was filtered through Celite and the filtrate was concentrated under reduced pressure. Methyl 2-acetoxy-5-aminobenzoate **248** was obtained as a light brown solid (394 mg, 90%) and was used in the next step without further purification but readily recrystallizes from cyclohexane/EtOAc. Mp. 107 ^{o}C (dec.), (Lit.,[37] 103-105 ^{o}C). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.31 (s, 3 H, C=OC*H*$_3$), 3.74 (s, 2 H, NH_2), 3.84 (s, 3 H, OC*H*$_3$), 6.82 (dd, 1 H, *J* = 8.4 Hz, 3 Hz, Ar-*H*), 6.88 (d, 1 H, *J* = 8.4 Hz, Ar-*H*), 7.28 (d, 1 H, *J* = 3 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 21.0 (C=O*C*H$_3$), 52.2 (O*C*H$_3$), 117.5 (Ar-*C*), 120.0 (Ar-*C*), 123.3 (Ar-*C*), 124.4 (Ar-*C*), 142.5 (Ar-*C*), 144.4 (Ar-*C*), 165.1 (*C*=O), 170.4 (*C*=O).

3.5.40. General Procedure for the Synthesis of Methyl Benzoate Amide Conjugates 145, 146, 249-253



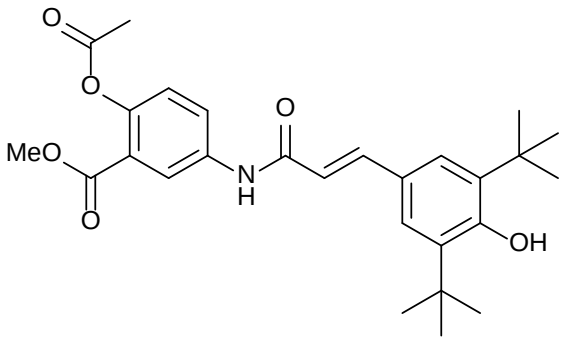
A solution of methyl 2-acetoxy-5-aminobenzoate **248** (124 mg, 591 µmol, 1.1 equiv.), appropriate carboxylic acid **8**, **70, 204-206**, **258** or **259** (537 µmol, 1 equiv.), EDC (123.5 mg, 599 µmol, 1.2 equiv.), and DMAP (8.2 mg, 67 µmol, 0.125 equiv.) in DCM (10 mL) was stirred under Ar overnight. The resulting reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography ($CHCl_3$ or Hexane/EtOAc, 3:1) to give the corresponding amide (**145**, **146**, **249-253**).

Methyl 2-acetoxy-5-(6-hydroxy-2,5,7,8-tetraethylchromane-2-carboxamido)benzoate **145**



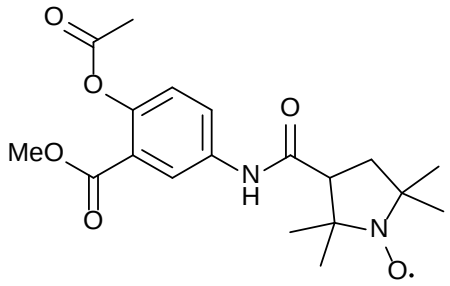
Beige solid (206.3 mg, 87%). Mp. 123-124 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 1.58 (s, 3 H, CH_3), 1.96 (m, 1 H, CH_2), 2.21 (s, 3 H, CH_3), 2.27 (s, 3 H, CH_3), 2.33 (s, 3 H, CH_3), 2.4 (s, 3 H, CH_3), 2.42 (m, 1 H, CH_2), 2.65 (m, 2 H, CH_2), 3.86 (s, 3 H, OCH_3), 4.32 (s, 1 H, N*H*), 7.05 (d, 1 H, *J* = 9 Hz, Ar-*H*), 7.75 (dd, 1 H, *J* = 9 Hz, 3 Hz, Ar-*H*), 8.08 (d, 1 H, *J* = 3 Hz, Ar-*H*), 8.38 (s, 1 H, O*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 11.3 (*C*H_3), 12.1 (*C*H_3), 12.3 (*C*H_3), 20.4 (*C*H_3), 20.9 (*C*H_3), 24.2 (*C*H_2), 29.5 (*C*H_2), 52.4 (O*C*H_3), 118.0 (Ar-*C*), 119.1 (Ar-*C*), 121.6 (Ar-*C*), 121.9 (Ar-*C*), 122.4 (Ar-*C*), 123.5 (Ar-*C*), 124.4 (Ar-*C*), 124.8 (Ar-*C*), 135.3 (Ar-*C*), 143.9 (Ar-*C*), 146.0 (Ar-*C*), 146.8 (Ar-*C*), 164.5 (*C*=O), 169.8 (*C*=O), 172.9 (*C*=O). HRMS (ES): *m*/*z* (%) = 442.1789 (35) $[M + H]^+$; calcd. for $C_{24}H_{27}NO_7$: 441.1788; found 441.1788. ATR-FTIR: υ_{max}= 3478 (m, O-H), 3364 (m, N-H), 2929 (m, ArC-H), 1762, 1728 (s, C=O), 1671 (s, C=O), 1531 (s, C=O), 1184 and 1078 (s, C-O) cm^{-1}.

Methyl (*E*)-2-acetoxy-5-(3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)acrylamido)benzoate **146**



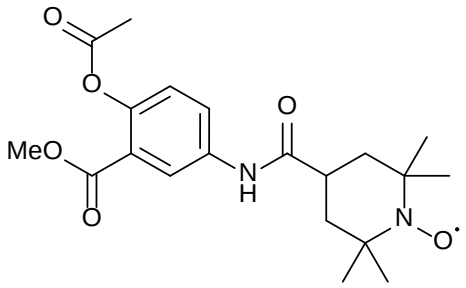
Beige solid (233.5 mg, 93%). Mp. 181-182 °C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.48 (s, 18 H, C(C*H*$_3$)$_3$), 2.36 (s, 3 H, C*H*$_3$), 3.86 (s, 3 H, OC*H*$_3$), 5.52 (s, 1 H, N*H*), 6.35 (d, 1 H, *J* = 15.6 Hz, CH=C*H*), 7.07 (d, 1 H, *J* = 7.8 Hz, Ar-*H*), 7.41 (d, 3 H, Ar-*H*), 7.72 (d, 1 H, *J* = 15.6 Hz, C*H*=CH), 7.96 (d, 1 H, *J* = 7.8 Hz, Ar-*H*), 8.13 (s, 1 H, O*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 21 (*C*H$_3$), 30.12 (C(*C*H$_3$)$_3$), 34.36 (*C*(CH$_3$)$_3$), 52.3 (O*C*H$_3$), 116.83 (Ar-*C*), 122.65 (Ar-*C*), 123.2 (Ar-*C*), 124.3 (Ar-*C*), 125.16 (Ar-*C*), 125.41 (CH=*C*H), 125.8 (Ar-*C*), 136.28 (Ar-*C*), 136.38 (*C*H=CH), 144.02 (Ar-*C*), 146.5 (Ar-*C*), 156.07 (Ar-*C*), 164.5 (*C*=O), 164.57 (*C*=O), 170.23 (*C*=O). HRMS (ES): *m*/*z* (%) = 468.2339 (100) $[M + H]^+$; calcd. for $C_{27}H_{33}NO_6$: 467.2308; found 467.2308. ATR-FTIR: υ_{max}= 3623 (m, br, O-H), 3305 (m, br, N-H), 2954 (m, ArC-H), 1726 (s, C=O), 1621 (s, C=O), 1540 (s, C=O), 1185 (s, C-O) cm^{-1}.

Methyl 2-acetoxy-5-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-carboxamido)benzoate **249**



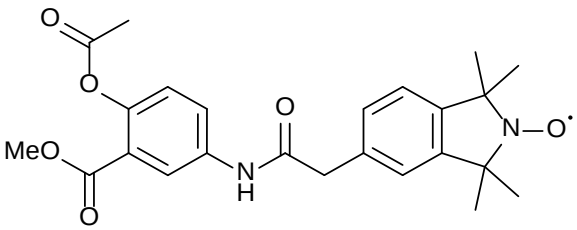
Yellow solid (170 mg, 84%). Mp. 69-70 °C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 2.37 (s, 3 H, C=OC*H*$_3$), 3.89 (s, 3 H, OC*H*$_3$), 7.12 (br, s, 1 H, Ar-*H*), 7.84 (br, s, 1 H, Ar-*H*), 8.04 (br, s, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 378.1744 (30) $[M + H]^+$; calcd. for $C_{19}H_{25}N_2O_6^{\bullet}$: 377.1713; found 377.1711. ATR-FTIR: υ_{max}= 3330 (m, N-H), 2975 (m, ArC-H), 1768 (s, C=O), 1726 (s, C=O), 1540 (s, C=O), 1366 (s, C-N), 1183 (s, C-O) cm^{-1}.

Methyl 2-acetoxy-5-(2,2,6,6-tetramethylpiperidin-1-yloxyl-4-carboxamido)benzoate **250**



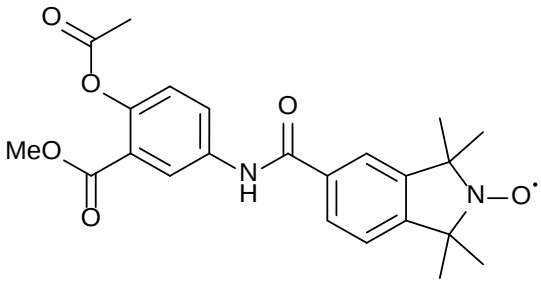
Light reddish-brown solid (156 mg, 74%). Mp. 205 ^{o}C (dec.). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 2.39 (s, 3 H, C=OCH_3), 3.85 (s, 3 H, OCH_3), 7.28 (br, dd, 1 H, Ar-*H*), 7.73 (br, d, 1 H, Ar-*H*), 8.14 (br, d, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 392.1927 (15) $[M + H]^+$, 414.1760 (100) $[M + Na]^+$; calcd. for $C_{20}H_{27}N_2O_6^{\bullet}$: 391.1869; found 391.1871. ATR-FTIR: υ_{max}= 3339 (m, N-H), 2977 (m, ArC-H), 1765, 1727 (s, C=O), 1691 (s, C=O), 1548 (s, C=O), 1176 (s, C-N), 1076 (s, C-O) cm^{-1}.

Methyl 2-acetoxy-5-(2-(1,1,3,3-tetramethylisoinolin-2-yloxyl-5-yl)acetamido) benzoate **251**



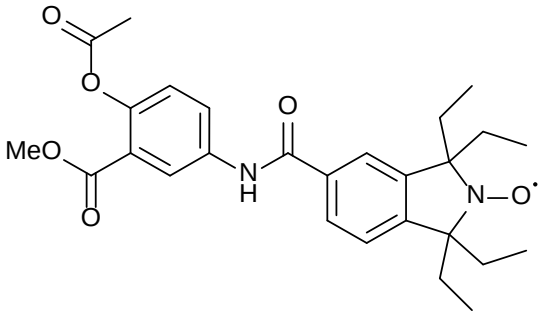
Yellow solid (224.2 mg, 95%). Mp. 215 ^{o}C (dec.). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 2.34 (s, 3 H, C=OCH_3), 3.85 (s, 3 H, OCH_3), 4.21 (br, s, 2 H, CH_2), 7.7.09 (br, s, 1 H, Ar-*H*), 7.86 (br, s, 1 H, Ar-*H*), 7.95 (br, s, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 440.1939 (30) $[M + H]^+$; calcd. for $C_{24}H_{27}N_2O_6^{\bullet}$: 439.1869; found 439.1869. ATR-FTIR: υ_{max}= 3273 (m, N-H), 2976 (m, ArC-H), 1760, 1729 (s, C=O), 1696 (s, C=O), 1548 (s, C=O), 1297 (s, C-N), 1191 and 1079 (s, C-O) cm^{-1}.

Methyl 2-acetoxy-5-(1,1,3,3-tetramethylisoinolin-2-yloxyl-5-carboxamido)benzoate **252**



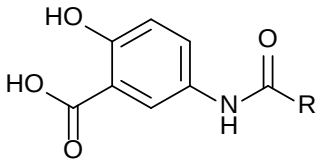
Yellow solid (190 mg, 83%). Mp. 122-124 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 2.3 (s, 3 H, C=OC*H*$_3$), 3.79 (s, 3 H, OC*H*$_3$), 7.05 (br, s, 1 H, Ar-*H*), 7.92 (br, s, 1 H, Ar-*H*), 8.09 (br, s, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 21.3 (C=O*C*H$_3$), 52.6 (O*C*H$_3$), 123.52 (Ar-*C*), 124.7 (Ar-*C*), 135.5 (Ar-*C*), 147.2 (Ar-*C*), 164.8 (*C*=O), 170.1 (*C*=O). HRMS (ES): *m*/*z* (%) = 426.1794 (10) $[M + H]^+$, 148.1606 (100) $[M + Na]^+$; calcd. for $C_{23}H_{25}N_2O_6^{\bullet}$: 425.1713; found 425.1716. ATR-FTIR: υ_{max}= 3376 (m, N-H), 2980 (m, ArC-H), 1735, 1725 (s, C=O), 1664 (s, C=O), 1522 (s, C=O), 1271 (s, C-N), 1226 and 1189 (s, C-O) cm^{-1}.

Methyl 2-acetoxy-5-(1,1,3,3-tetraethylisoinolin-2-yloxyl-5-carboxamido)benzoate **253**



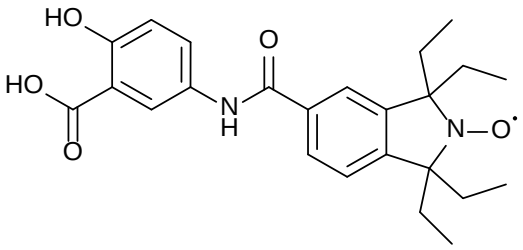
Yellow solid (209.5 mg, 81%). Mp. 122-124 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 2.41 (s, 3 H, C=OC*H*$_3$), 3.91 (s, 3 H, OC*H*$_3$), 7.17 (br, s, 1 H, Ar-*H*), 8.05 (br, s, 1 H, Ar-*H*), 8.22 (br, s, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 21.15 (C=O*C*H$_3$), 52.49 (O*C*H$_3$), 123.5 (Ar-*C*), 124 (Ar-*C*), 135.67 (Ar-*C*), 147.13 (Ar-*C*), 164.48 (*C*=O), 170.07 (*C*=O). HRMS (ES): *m*/*z* (%) = 481.2410 (20) $[M + H]^+$; calcd. for $C_{27}H_{33}N_2O_6^{\bullet}$: 481.2339; found 481.2341. ATR-FTIR: υ_{max}= 3369 (m, N-H), 2971 (m, ArC-H), 1743, 1719 (s, C=O), 1675 (s, C=O), 1534 (s, C=O), 1298 (s, C-N), 1219 and 1188 (s, C-O) cm^{-1}.

3.5.41. General Procedure for the Hydrolysis of Methyl Benzoate Amide Conjugates 249-253



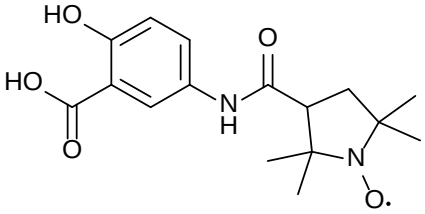
A solution of NaOH (3 mL, 1 M) was added to a solution of appropriate amide (**235-239**) in THF (5 mL) and the reaction mixture was stirred at RT overnight. THF was removed under pressure and the aqueous layer washed with DCM, then cooled in ice/H_2O bath and acidified (to pH 1) with HCl (2 M). The precipitate formed was isolated by filtration and purified by recrystallization from H_2O/MeOH to give the corresponding salicylic acid derivative (**141**, **254-257**).

2-Hydroxy-5-(1,1,3,3-tetraethylisoindolin-2-yloxyl-5-carboxamido)benzoic acid **141**



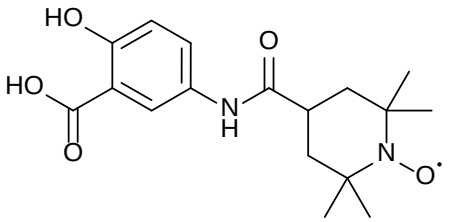
Yellow solid (209.5 mg, 81%). Mp. 208 ^oC (dec.). HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 426.2091 (45) $[M + H]^+$; calcd. for $C_{24}H_{29}N_2O_5^{\bullet}$: 425.2076; found 425.2081. ATR-FTIR: υ_{max}= 3295 (m, br, O-H), 2968 (m, ArC-H), 1686, 1637 (s, C=O), 1536 (s, C=O), 1167 (s, C-O) cm^{-1}.

2-Hydroxy-5-(2,2,5,5-tetramethylpyrrolidin-1-yloxyl-3-carboxamido)benzoic acid **254**



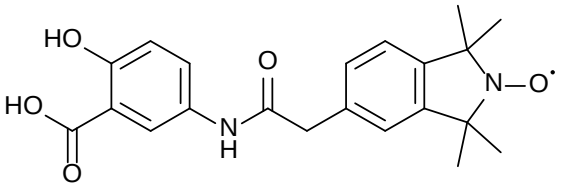
Yellow solid (170 mg, 84%). Mp. 171 ^{o}C (dec.). HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 321.1452 (20) [M], 322.1510 (20) $[M + H]^+$, 323.1590 (100) $[M + 2H]^+$; calcd. for $C_{16}H_{21}N_2O_5^{\bullet}$: 321.1450; found 321.1452. ATR-FTIR: υ_{max}= 3200 (m, br, O-H), 3921 (m, ArC-H), 1657 (s, C=O), 1547 (s, C=O), 1225 (s, C-O) cm^{-1}.

2-Hydroxy-5-(2,2,6,6-tetramethylpiperidin-1-yloxyl-4-carboxamido)benzoic acid **255**



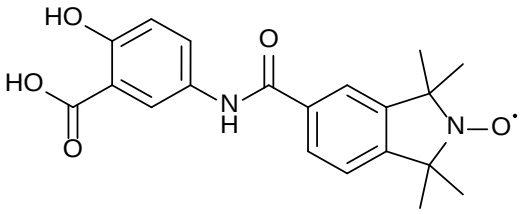
Light reddish-brown solid (156 mg, 74%). HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 336.1729 (15) $[M + H]^+$, 337.1753 (100) $[M + 2H]^+$; calcd. for $C_{17}H_{23}N_2O_5^{\bullet}$: 335.1607; found 335.1607. ATR-FTIR: υ_{max}= 3200 (m, br, O-H), 3921 (m, ArC-H), 1657 (s, C=O), 1547 (s, C=O), 1225 (s, C-O) cm^{-1}.

2-Hydroxy-5-(2-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)acetamido)benzoic acid **256**



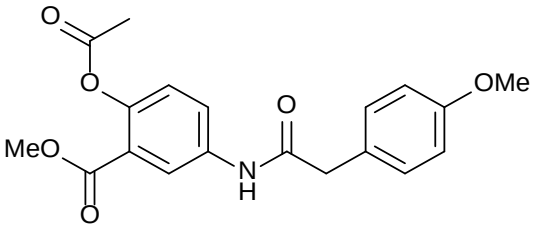
Yellow solid (224.2 mg, 95%). Mp. 115 ^{o}C (dec.). HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 385.1834 (20) $[M + 2H]^+$; calcd. for $C_{21}H_{23}N_2O_5^{\bullet}$: 383.1607; found 383.1611. ATR-FTIR: υ_{max}= 3263 (m, br, O-H), 2923 (m, ArC-H), 1674, 1647 (s, C=O), 1561 (s, C=O), 1208 (s, C-O) cm^{-1}.

2-Hydroxy-5-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carboxamido)benzoic acid **257**



Yellow solid (190 mg, 83%). Mp. 207 °C (dec.). HPLC purity (>95%). HRMS (ES): *m*/*z* (%) = 392.3146 (100) $[M + Na]^+$; calcd. for $C_{20}H_{21}N_2O_5^{\bullet}$: 369.1450; found 369.1460. ATR-FTIR: υ_{max}= 3100 (m, br, O-H), 2954 (m, ArC-H), 1676, 1644 (s, C=O), 1565 (s, C=O), 1187 (s, C-O) cm^{-1}.

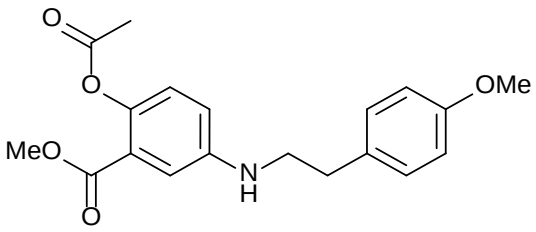
3.5.42. Synthesis of Methyl 2-acetoxy-5-(2-(4-methoxyphenyl acetamido)benzoate 264



A solution of methyl 2-acetoxy-5-aminobenzoate **248** (124 mg, 591 µmol, 1.1 equiv.), 2-(4-methoxyphenyl)acetic acid **265** (89 mg, 537 µmol, 1 quiv.), EDC (123.5 mg, 599 µmol, 1.2 equiv.), and DMAP (8.2 mg, 67 µmol, 0.125 equiv.) in DCM (10 mL) was stirred under Ar overnight. The resulting reaction mixture was diluted (DCM, 100 mL), washed brine (40 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography ($CHCl_3$ or Hexane/EtOAc, 3:1) to give methyl 2-acetoxy-5-(*N*-(4-methoxyphenylacetamide))benzoate ester **264** as a white solid (155, 81%). Mp. 49-51 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 2.32 (s, 3 H, C=OC*H*$_3$), 3.69 (s, 2 H, C*H*$_2$), 3.839 (s, 3 H, OC*H*$_3$), 3.84 (s, 3 H, OC*H*$_3$), 6.95 (d, 2 H, *J* = 8.4 Hz, Ar-*H*), 7.02 (d, 1 H, *J* = 9 Hz, Ar-*H*), 7.13 (s, 1 H, N*H*), 7.24 (d, 2 H, *J* = 8.4 Hz, Ar-*H*), 7.84 (m, 2 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 20.9 (C=O*C*H$_3$), 34.9 (C*H*$_2$), 52.3 (O*C*H$_3$), 55.2 (O*C*H$_3$), 114.8 (Ar-*C*), 122.4 (Ar-*C*), 123.3 (Ar-*C*), 124.4 (Ar-*C*), 125.1 (Ar-*C*), 125.9 (Ar-*C*), 130.7 (Ar-*C*), 135.6 (Ar-*C*), 146.7 (Ar-*C*), 159.3 (Ar-*C*), 164.5 (*C*=O), 169.6 (*C*=O), 169.8 (*C*=O). HRMS (ES): *m*/*z* (%) = 358.1286 (30) [M + H], 380.1102 (100) $[M + Na]^+$; calcd. for $C_{19}H_{19}NO_6$: 357.1212; found 357.1212.

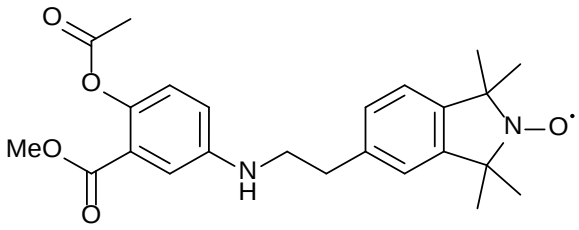
ATR-FTIR: υ_{max}= 3371 (m, N-H), 2934 (w, Ar C-H), 1718 (s, C=O), 1698 (s, C=O), 1540 (s, C=O), 1296 (s, C-N), 1191 (s, C-O) cm^{-1}.

3.5.43. Synthesis of Methyl 2-acetoxy-5-((4-methoxyphenethyl) amino)benzoate 266



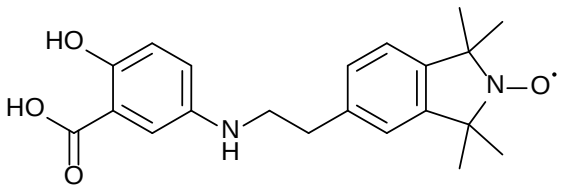
Anhydrous acetic acid (40 µL, 700 µmol, 5 equiv.) was added dropwise to a suspension of $NaBH_4$ (27 mg, 707 µmol, 5.05 equiv.) and methyl 2-acetoxy-5-(2-(4-methoxyphenylacetamido)benzoate **264** (50 mg, 140 µmol, 1 equiv.) in dry dioxane (3 mL). The resulting reaction mixture was refluxed for 25 min and then allowed to return to RT. H_2O was added and the resulting aqueous solution was extracted with EtOAc (30 mL x 4). The combined organic layer was washed with brine (30 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was purified silica gel column chromatography (Hexane/EtOAc, 3:1) to give methyl 2-acetoxy-5-((4-methoxyphenethyl)amino)benzoate **266** as a white solid (32 mg, 66%). Mp. 49-51 ^oC (Lit.,[40] 49-50 ^oC). ^1H NMR ($CDCl_3$, 600 MHz): δ = 2.31 (s, 3 H, C=OCH_3), 2.86 (t, 2 H, *J* = 6.6 Hz, CH_2), 3.36 (t, 2 H, *J* = 6.6 Hz, CH_2), 3.8 (s, 3 H, OCH_3), 3.84 (s, 3 H, OCH_3), 6.73 (dd, 2 H, *J* = 9 Hz, 3 Hz, Ar-*H*), 6.87 (m, 3 H, Ar-*H*), 7.13 (d, 2 H, *J* = 8.4 Hz, Ar-*H*), 7.18 (d, 1 H, *J* = 3 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 20.2 (C=O*C*H$_3$), 34.9 (CH_2), 52.3 (O*C*H$_3$), 55.4 (O*C*H$_3$), 114.8 (Ar-*C*), 122.4 (Ar-*C*), 123.3 (Ar-*C*), 124.4 (Ar-*C*), 125.1 (Ar-*C*), 125.9 (Ar-*C*), 130.7 (Ar-*C*), 135.5 (Ar-*C*), 146.7 (Ar-*C*), 159.3 (Ar-*C*), 164.5 (*C*=O), 169.6 (*C*=O). HRMS (ES): *m*/*z* (%) = 344.1455 (50) $[M + H]^+$; calcd. for $C_{19}H_{21}NO_5$: 343.1420; found 343.1422. ATR-FTIR: υ_{max}= 3393 (m, N-H), 2951 (w, Ar C-H), 1758 (s, C=O), 1718 (s, C=O), 1189 (s, C-O) cm^{-1}.

3.5.44. Synthesis of Methyl 2-acetoxy-5-((2-(1,1,3,3-tetramethyl isoindolin-2yloxyl-5-yl)amino)benzoate 267



Anhydrous acetic acid (42 µL, 728 µmol, 5 equiv.) was added dropwise over 10 min to a suspension of $NaBH_4$ (28 mg, 735 µmol, 5.05 equiv.) and methyl 2-acetoxy-5-(2-(1,1,3,3-tetraethylisoinolin-2-yloxyl-5-yl)acetamido)benzoate **251** (64 mg, 146 µmol, 1 equiv.) in dry dioxane (3 mL). The resulting reaction mixture was refluxed for 30 min and then allowed to return to RT. H_2O was added and the resulting aqueous solution was extracted with EtOAc (30 mL x 4). The combined organic layer was washed with brine (30 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was purified silica gel column chromatography (Hexane/EtOAc, 3:1) to give methyl 2-acetoxy-5-((2-(1,1,3,3-tetramethylisoindolin-2yloxyl-5-yl)amino)benzoate **267** as yellow solid (42 mg, 68%). Mp. 85-87 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 600 MHz): δ = 2.31 (s, 3 H, C=OCH_3), 3.45 (br, s, 2 H, CH_2), 3.85 (s, 3 H, OCH_3), 6.76 (br, s, 1 H, Ar-*H*), 6.92 (br, s, 1 H, Ar-*H*), 7.08 (br, s, 1 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 426.2111 (40) $[M + H]^+$; calcd. for $C_{24}H_{29}N_2O_5^{\bullet}$: 425.2076; found 425.2076. ATR-FTIR: υ_{max}= 3371 (m, N-H), 2972 (w, Ar C-H), 1746 (s, C=O), 1715 (s, C=O), 1195 (s, C-O) cm^{-1}.

3.5.45. Synthesis of 2-Hydroxy-5-((2-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethyl)amino)benzoic acid 142



NaOH (2 mL, 0.5 M) was added to a solution of methyl 2-acetoxy-5-((2-(1,1,3,3-tetramethyl isoindolin-2yloxyl-5-yl)amino)benzoate **267** (40 mg, 94 µmol) in MeOH (2 mL) and the reaction mixture was stirred at RT overnight. MeOH was removed under pressure and the aqueous layer washed with DCM (5 mL), then cooled in

ice/H_2O bath and acidified (to pH 3) with HCl (2 M). The precipitate formed was isolated by filtration and purified by recrystallization from H_2O/MeOH to give 2-hydroxy-5-((2-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethyl)amino)benzoic acid **142** as brownish yellow solid (25 mg, 72%). Mp. 200 ^oC (dec.). HRMS (ES): *m/z* (%) = 370.1847 (15) $[M + H]^+$; calcd. for $C_{21}H_{25}N_2O_4^{\bullet}$: 369.1814; found 369.1817. ATR-FTIR: υ_{max}= 3500-2500 (m, br, O-H), 2975 (m, Ar C-H), 1681 (s, C=O), 1215 (s, C-O) cm^{-1}.

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Chapter 4: Synthesis of L-Dopa and Ritalin Nitroxide Analogues

4.1 Background

L-Dopa **147** and Ritalin (or Methylphenidate, MPH) **148** (**Figure 4.1**) are two sympathomimetic drugs identified as the standard-of-care treatment for Parkinson's disease (PD) and attention deficit hyperactivity disorder (ADHD) respectively.[1-5] Like most sympathomimetic amine stimulants, L-Dopa and Ritalin share a common phenethylamine core. They exert their therapeutic action by mainly altering or mimicking the sympathetic nervous system functions. PD is a chronic, progressive neurodegenerative disorder caused primarily by the loss of dopamine-producing cells (dopaminergic neurons) within the substantia nigra of the central nervous system.[4,6,7] Dopamine **152** is a neurotransmitter that regulates diverse, normal physiological functions (movement, reward, motivation, and learning).

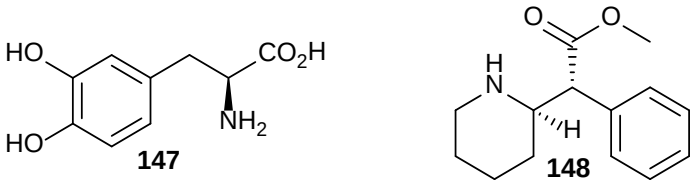
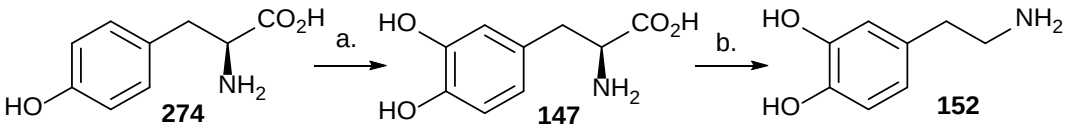


Figure 4.1. L-Dopa **147** and methylphenidate **148** chemical structures.

The characteristic PD motor symptoms such as tremor, rigidity and bradykinesia (slowness of movement) are attributed to dopamine depletion in the substantia nigra of the central nervous system. Dopamine **152** biosynthesis from L-tyrosine **274** takes place in the cytosol catecholaminergic neurons (**Scheme 4.1**).[8]



Scheme 4.1. Biosynthesis of dopamine **152** from L-tyrosine **274**: a. tyrosine hydroxylase; b. AADC.

Enzymes such as tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC) successively catalyze the dopamine biosynthetic pathway. Since the pathogenesis of PD is mainly linked to the loss of dopaminergic neurons (which in turn leads to decreased amounts of available physiological dopamine), a rational strategy to manage PD should involve a direct dopamine replacement therapy. However, dopamine does not cross the blood brain barrier. For this reason, L-Dopa, a biosynthetic precursor to dopamine that readily crosses the blood brain barrier, is used for PD management.

ADHD is a common childhood behavioural disorder that affects 5–10% of the general population.[9,10] It is characterized by a number of behavioural symptoms such as small attention span, impulsivity, and hyperactivity. Ritalin relieves ADHD-behavioural symptoms by blocking the reuptake of dopamine and noradrenaline by presynaptic neurons thereby increasing the intrasynaptic concentrations of dopamine and noradrenaline. [11-13]

Despite the therapeutic benefits of L-Dopa and Ritalin, long-term use of these sympathomimetic agents often leads to side effects such as dyskinesia (difficulty in performing voluntary movements, in PD) and oxidative brain damage (in ADHD).[14-16] Notably, oxidative stress is identified as a major contributor to the pathology of PD and ADHD as well as their associated drug-induced cytotoxicity.[8,17-22] In nigral cells, dopamine spontaneously oxidizes to a dark pigment known as neuromelanin.[8] The dopamine-autoxidation process also generates free radicals (such as hydroxyl and superoxide radicals) and neurotoxic semiquinone and quinone metabolites. The dopamine-derived quinoids are strong electrophiles which, when attacked by nucleophiles such as the sulfhydryl group, induce damage to biomolecules like proteins in the midbrain.[8,23] Redox active metals found in large quantities in the substantia nigra of PD patients also promote dopamine-induced autoxidative neuronal damage.[24] Iron for instance induces cellular damage by forming reactive complexes with the catechol group of dopamine and dopamine-metabolites. The implication of oxidative stress in PD pathology is further supported by reports of decreased levels of reduced-glutathione (GSH) along with the increase in the activities of GSH-associated metabolizing enzymes including GSH peroxidase observed in the substantia nigra of PD patients.[25-29] Glutathione is natural antioxidant

that protects dopaminergic neurons by detoxifying ROS generated in the midbrain. Dopaminergic neurons in PD patients also display a high rate of basal lipid peroxidation.[30,31] This, along with depleted levels of nigral endogenous antioxidant enzymes (such as SOD and catalase) evidently support the role of oxidative stress in PD pathogenesis.[30-34]

Similarly, Ritalin-induced oxidative stress is well documented.[16,35-38] Thus, the continuing vulnerability of dopaminergic neurons to both drug-induced and pathological oxidative stress evidently suggest a clear need for an antioxidant therapeutic intervention. In a recent PD model study, the antioxidant nitroxide TEMPOL was shown to protect dopaminergic mesencephalic cells from 6-hydroxydopamine (a dopamine metabolite)-induced apoptosis. As part of a wider strategy towards the use of the antioxidant nitroxides as a potential remedy for oxidative stress-related diseases, this chapter further explores the antioxidant capacity of stable nitroxide compounds in neurodegenerative disorders, particularly PD and ADHD. The aim here is to use the pharmacophore hybridization strategy to design and synthesize new hybrid dual acting nitroxide-based anti-PD and anti-ADHD agents **149** and **150**, by overlapping the functional components of L-Dopa and MPH with stable antioxidant nitroxides (**Figure 4.2**).

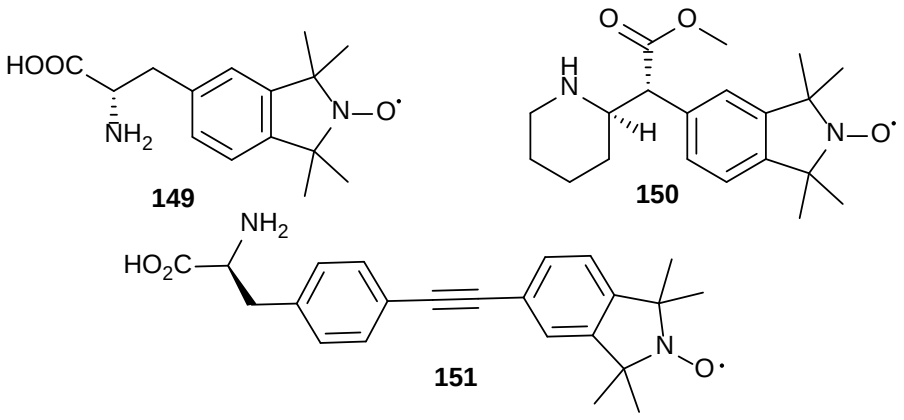


Figure 4.2. Chemical structures of merged target compounds: L-Dopa TMIO **149**, Ritalin TMIO **150** and the rigid alkyne-linked α-amino acid **151**.

In addition to the L-Dopa and Ritalin nitroxide analogues **149** and **150**, the rigid alkyne-linked α-amino acid nitroxide **151** is the final target compound in this chapter. The synthesis of compound **151** will be carried out by taking advantage of

the Schöllkopf's approach that would be employed in the asymmetric synthesis of the target L-Dopa nitroxide analogue **149**. Unlike the L-Dopa and Ritalin nitroxides **149** and **150**, compound **151** is designed as a potential site-directed spin label (SDSL) for studying the structure and conformational dynamics of peptides and proteins. Furthermore, **151** could also be used as a tool for monitoring ROS-induced cellular oxidative stress in diseases such as PD by both EPR and fluorescence techniques.

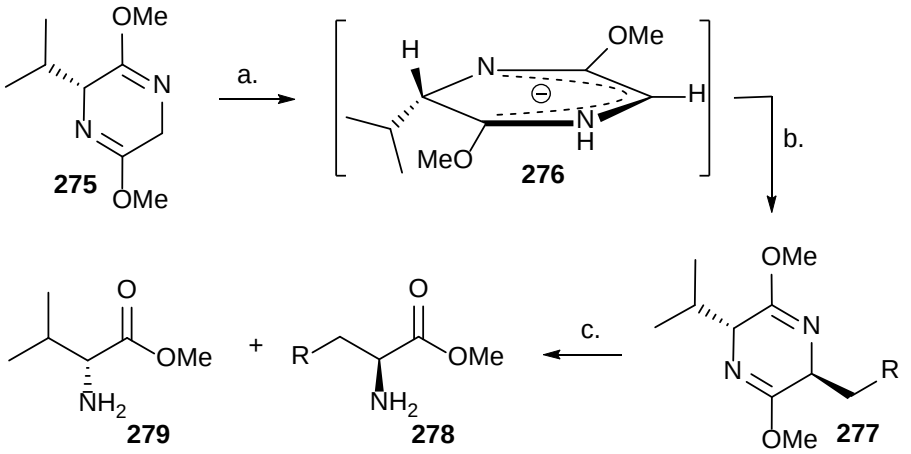
4.2 Results and Discussion

4.2.1 Schöllkopf's Synthesis of Merged L-Dopa-TMIO 149

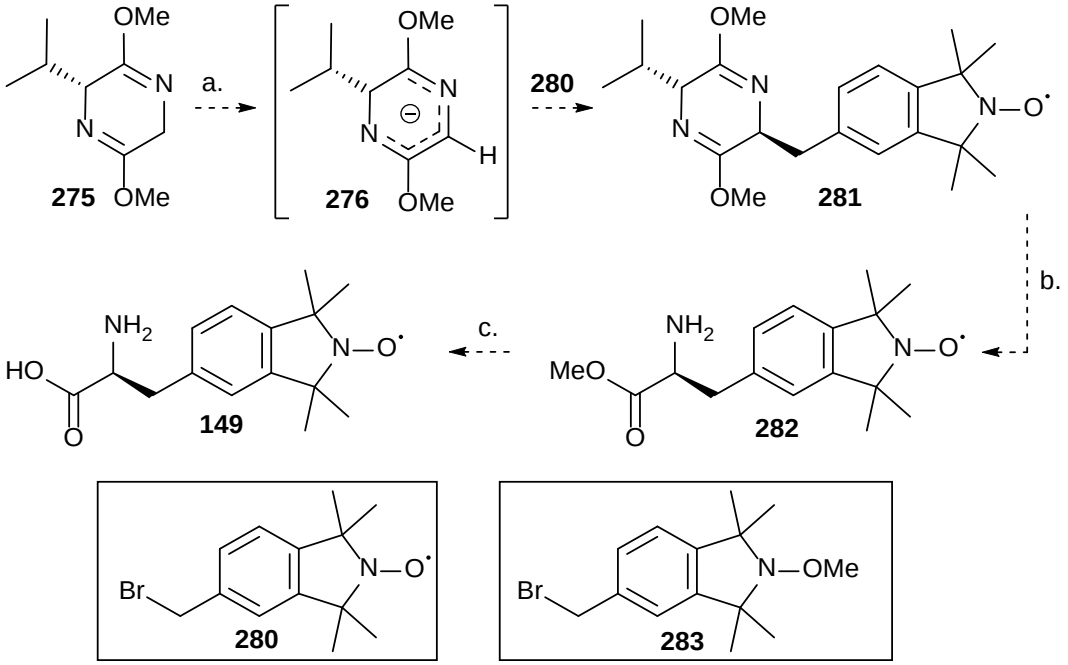
Since D-dopa is not a substrate for the AADC enzyme (which metabolizes L-Dopa to dopamine),[39-41] L-Dopa is administered as a single enantiomer in dopamine replacement therapy. Hence, the challenge herein is to employ a synthetic approach that will predominantly afford the target compound **149** in its optically active L-isomer form. Though a variety of methods are available to asymmetrically prepare chiral nonproteogenic α-amino acids,[42-44] Schöllkopf's approach was employed in the synthesis of the target L-Dopa nitroxide.[45]

The Schöllkopf methodology is an asymmetric nucleophilic derivatization approach used to prepare chiral amino acids. The asymmetric induction for the Schöllkopf approach is usually greater than 95%.[45-48] The method uses bis-lactim ethers such as (2*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine **275** as precursors to a masked glycine synthetic equivalents (enolates). Bis-lactim ether substrates, also referred to as Schöllkopf chiral auxiliaries, are commonly prepared by cyclizing glycine with a chiral auxiliary. α-Amino acids such as *R*-valine are the most common chiral auxiliaries employed.[49] The bis-lactim ether **275** has two acidic protons at C_2. Deprotonation at C_2 by a strong base generates the planar masked glycine enolate **276** (**Scheme 4.2**). The *in situ* generated carbanion **276** is then alkylated diastereoselectively to give the alkyl dihydropyrazine intermediate **277**. In the presence of an electrophile, the side chain (isopropyl group) of **276** acts as a chiral inducing centre that directs electrophilic attack from the opposite side of the planar enolate **276**. The bis-lactim ether **275** also contains two hydrolyzable sites (at the two

nitrogen atoms). Following alkylation, the dihydropyrazine ring can be readily hydrolyzed to furnish an optically pure free amino methyl ester **178** along with the methyl D-valinate chiral auxiliary **279**. **Scheme 4.3** below outlines the proposed synthetic route for the synthesis of the target L-Dopa nitroxide **149** via the Schöllkopf's approach.



Scheme 4.2. The Schöllkopf's approach for asymmetric synthesis of chiral α-amino acids. Reagents and conditions**:** a. *n*BuLi, -78 ^{o}C; b. RCHX (X = Br, Cl or I); c. HCl.

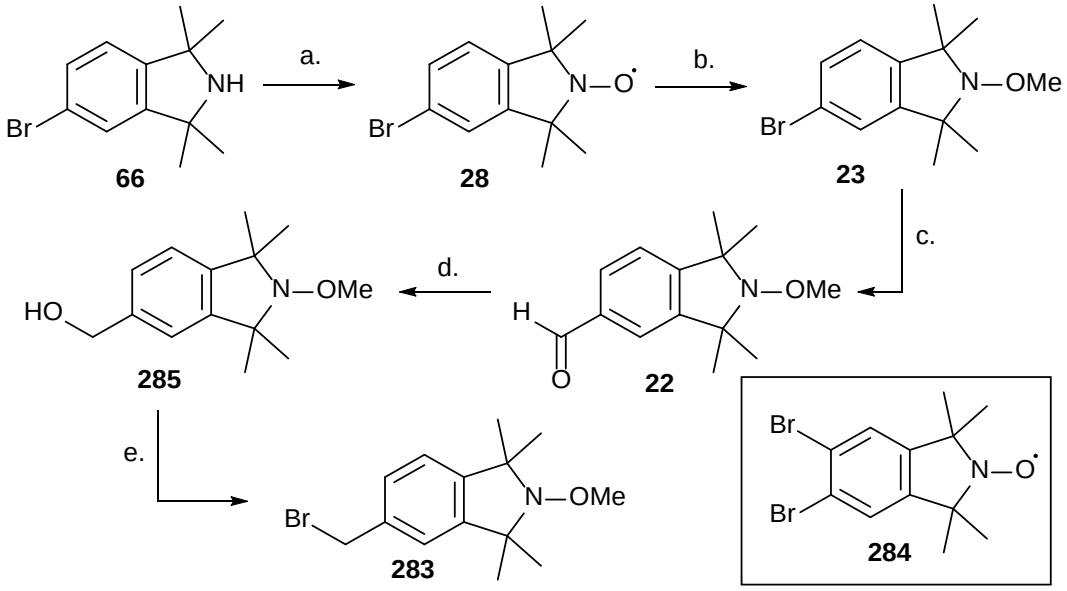


Scheme 4.3. Proposed synthetic route to L-Dopa nitroxide **149**. Reagents and conditions: a. *n*BuLi, THF, -78 ^{o}C; b. HCl, THF; c. LiOH, THF.

However, in order to conveniently assess the diastereoselectivity of this approach by ^{1}H NMR spectroscopy, the alkylation of bis-lactim ether **275** was first attempted with the methoxyamine derivative **283** of the benzyl bromide nitroxide **280**.

4.2.1.1 Synthesis of Benzyl Bromide Methoxyamine 283

The benzyl bromide methoxyamine **283** was synthesized in seven steps from bromoamine precursor **66** as depicted below in **Scheme 4.4**. The bromoamine intermediate **66** was obtained in three steps from phthalic anhydride **167** by following the previous literature procedures discussed in **Chapter 2**.[50] Bromoamine **66** was oxidized to the corresponding nitroxide **28** under mild *m*CPBA oxidation conditions. Compound **28** was obtained almost quantitatively as yellow solid following purification by trituration with hexane to remove the small (less than 5%) 5,6-dibromo nitroxide impurity **284**, which is usually formed during the bromination step. All characterization data obtained for **28** matched that previously reported in the literature.[50]



Scheme 4.4. Synthesis of benzyl bromide *N*-methoxyamine **283**. Reagents and conditions: a. *m*CPBA, DCM, 0 ^{o}C-RT, 2 h, 87%; b. $FeSO_4.7H_2O$, H_2O_2, DMSO, RT, 1 h, 93%; c. *n*BuLi, THF, -78 ^{o}C, 15 min, then DMF, 3 h, 96%; d. $NaBH_4$, MeOH, 0 ^{o}C, 1.5 h, 97%; e. MsCl, TEA, 0 ^{o}C, 2 h, THF, Ar, then LiBr, Me_2CO, reflux, 2 h, 81%.

Since the nitroxide moiety can react with *n*-butyllithium to give the hydroxylamine, amine and alkoxyamine derivatives,[51,52] it was necessary to first protect the 5-bromonitroxide **28** prior to subjecting it to lithiation conditions. Thus **28** was converted to its methoxyamine derivative **23** by reacting it *in situ* with methyl radicals formed by the typical Fenton reaction of hydrogen peroxide and iron (II) sulfate with DMSO.[53] The methoxy protons of **23** were observed as a singlet at 3.8 ppm in the ^{1}H NMR spectrum. Other spectroscopic data obtained for **23** matched that reported in the literature.[53,54]

To synthesize the formyl derivative **22**, bromomethoxyamine **23** was first lithiated with *n*-butyllithium and subsequently quenched with DMF to furnish formyl **22** quantitatively. Evidence to support this transformation was obtained by the presence of the characteristic formyl proton signal as a singlet at 9.98 ppm in the ^{1}H NMR spectrum of **22**. A strong C=O absorption was also observed at 1690 cm^{-1} in the FTIR spectrum of **22**. The identity of **22** was further confirmed by the presence of the molecular ion plus proton $[M + H]^+$ at m/z 234 in the HRMS spectrum. The purity of **22** was assessed by analytical HPLC to be >95%.

The formyl methoxyamine **22** was then reduced by sodium borohydride to the corresponding benzyl alcohol **285** in 97% yield. The loss of the formyl proton signal at 9.98 ppm as well as the appearance of the benzylic CH_2 proton signal as a singlet at 4.68 ppm in the ^{1}H NMR spectrum of the isolated product supported the formation of **285**. In the FTIR spectrum of **285**, the presence of a strong-broad O-H absorption at 3393 cm^{-1} and the absence of a strong formyl C=O stretch at 1690 cm^{-1} further supported this successful transformation. The HRMS analysis also confirmed the identity of compound **285** with the molecular ion plus proton $[M + H]^+$ peak at m/z 326. Compound **285** was determined to be >95% pure by HPLC analysis.

In general, primary alcohols are not good leaving groups in bimolecular nucleophilic substitution reactions with halides. A common approach around this involves first converting the hydroxyl group into a good leaving sich as a mesylate, which is in turn readily substituted with the desired halide. Following a similar approach, compound **285** was first converted to the methanesulfonate ester intermediate by reacting with methanesulfonyl chloride in the presence of triethylamine. The

mesylate was then reacted *in situ* with lithium bromide to furnish compound **283**. Although TLC analysis of the reaction mixture (after two hours) showed complete consumption of the starting material **285** and the formation of a single new component, analysis of the isolated product by HPLC, HRMS and ^{1}H NMR spectroscopy revealed a two component mixture of the desired benzyl bromide **283** and its chloride derivative **286** (≤ 11%, **Figure 4.3**). The unexpected formation of the benzyl chloride **286** side-product could be explained by competing nucleophilic substitution of the mesylate by chloride ions (produced during *in situ* generation of the mesylate).

283 **286**

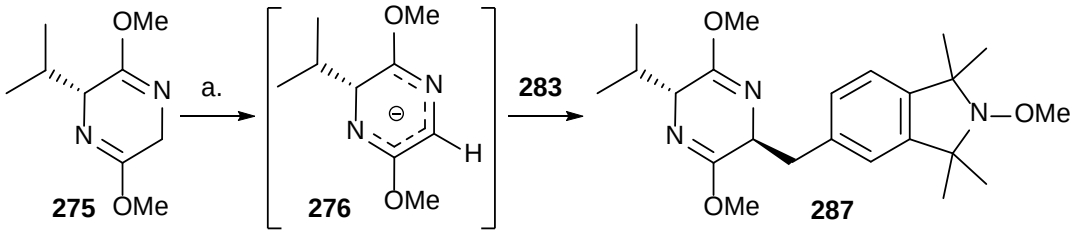
Figure 4.3. Chemical structures of benzyl bromide **283** and benzyl chloride **286** TMI methoxyamines.

Qi and co-workers also reported similar nucleophilic substitution of a series of benzylic sulfonate esters by chloride ions in modest yields.[55] However, since bromides are better nucleophiles than their chloride counterparts, the desired benzylic bromide **283** was obtained exclusively when the benzylic halide mixture was refluxed in acetone in the presence of excess lithium bromide for two hours. Compound **283** was obtained in high yield (81%) as a white solid following purification by flash column chromatography. The product isolated was characterized by HRMS, FTIR, ^{1}H and ^{13}C NMR spectroscopy. The molecular ion $[M + H]^{+}$, with the characteristic isotopic pattern for the presence of a single bromine atom, was observed at m/z 298/300 in the HRMS spectrum. The disappearance of a strong O-H absorption above 3393 cm^{-1} in the FTIR spectrum of **283** further supported the transformation. HPLC analysis of compound **283** showed a single component of >95% purity.

4.2.1.2 Alkylation of Schöllkopf's Bis-lactim Ether Chiral Auxiliary 275

With the benzyl bromide intermediate **283** in hand, the key step in the synthesis of the target L-Dopa-nitroxide analogue **149** was the subsequent asymmetric alkylation of the commercially available bis-lactim ether chiral auxiliary (2*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine **275** with benzyl bromide **283**. Freshly purchased compound **275** had a designated purity of 96% by GCMS. The ^{1}H NMR spectrum of **275** showed about 5% impurities. The impurities were reported to be the ethoxy derivatives of **275**, namely (2*R*)-2,5-dihydro-3-ethoxy-6-methoxy-2-isopropypiperazine and (2*R*)-2,5-dihydro-6-ethoxy-3-methoxy-2-isopropypiperazine.[49] These ethoxy contaminants are reportedly formed from an impurity in the triethyloxonium tetrafluoroborate reagent used in the synthesis of **275**. However, it was also stated that their presence does not affect the outcome of the alkylation step and they are readily separated from the desired product by chromatography.

Thus, the chiral auxiliary **275** was deprotonated at C_5 with equimolar amount of *n*-butyllithium to generate the enolate **276** (**Scheme 4.5**). As mentioned in the preceding section, one of the diastereotopic sides at C_5 of the planar enolate ion is strongly shielded by the bulky isopropyl group. This ensures electrophilic attack at C_5 to occur exclusively on the opposite face to the isopropyl group, thus resulting in the formation of the 2*R*, 5*S* diastereomer.



Scheme 4.5. Alkylation of bis-lactim ether **275** with benzyl bromide *N*-methoxyamine **283**. Reagents and conditions: a. *n*BuLi, -78 ^{o}C, 15 min, THF, then **283**, 3 h, 80%.

Upon quenching anion **276** with the benzyl bromide methoxyamine **283**, the single diastereomer **287** (2*S*, 5*R*) was obtained in high yield (after purification by column

chromatography). The diastereomeric purity of the isolated product **287** was confirmed by ^{1}H NMR spectroscopy to be >99%. The single proton of the central carbon of the isopropyl group was shown as a multiplet at 2.11 ppm while the benzylic protons (CH_2) were observed at 3.07 ppm. Three distinct aromatic protons were also observed at 7.11 and 7.26 ppm. This was consistent with the presence of the three protons of the isoindoline aromatic ring.

The dihydropyrazine proton of **287** labelled $\mathbf{H_a}$ in **Figure 4.4** below was also observed as a doublet at 3 ppm. This was, interestingly, much further upfield than the $\mathbf{H_a}$ proton of the chiral auxiliary starting material **275** which was observed at 4 ppm. As was the case with some benzyl-alkylated dihydropyrazines reported in the literature,[47,56] such marked difference in chemical shift suggests that compound **287** probably adopts an "aryl-inside" conformation (**287a** in **Figure 4.4**). In this conformation, the $\mathbf{H_a}$ proton of **287** is situated in the shielding cone of the isoindoline aromatic ring.

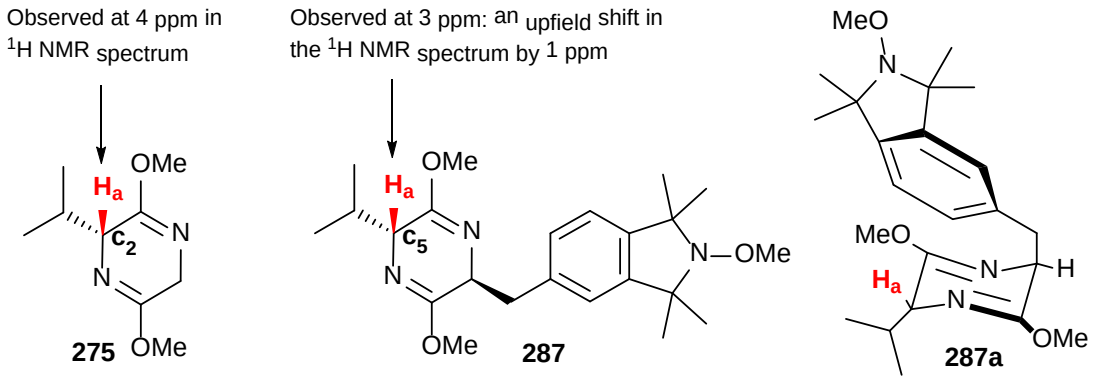


Figure 4.4. Chemical structure of **275**, **287** and the aryl-inside conformation of **287** (**287a**).

Good evidence to support the "aryl-inside" conformation of **287** was obtained from ^{1}H NMR ROESY analysis (**Figure 4.5**) which showed a clear spatial correlation between the dihydropyrazine ring $\mathbf{H_a}$ proton at 3 ppm and the isoindoline aromatic protons at 7.11 and 7.26 ppm (as highlighted in the ROESY spectrum **Figure 4.5**). Such a correlation also indicates that the isopropyl group and the isoindoline fragment are *anti* to each other with reference to the dihydropyrazine ring

plane. The evidence from ROESY spectrum is however inconclusive because the highlighted spatial correlation with the isoindoline aromatic protons could as well arise from the benzylic protons at 3.07 ppm which overlap the $\mathbf{H_a}$ proton signal at 3 ppm. Other characterization data obtained to support the identity of **287** included HRMS, FTIR and ^{13}C NMR spectra.

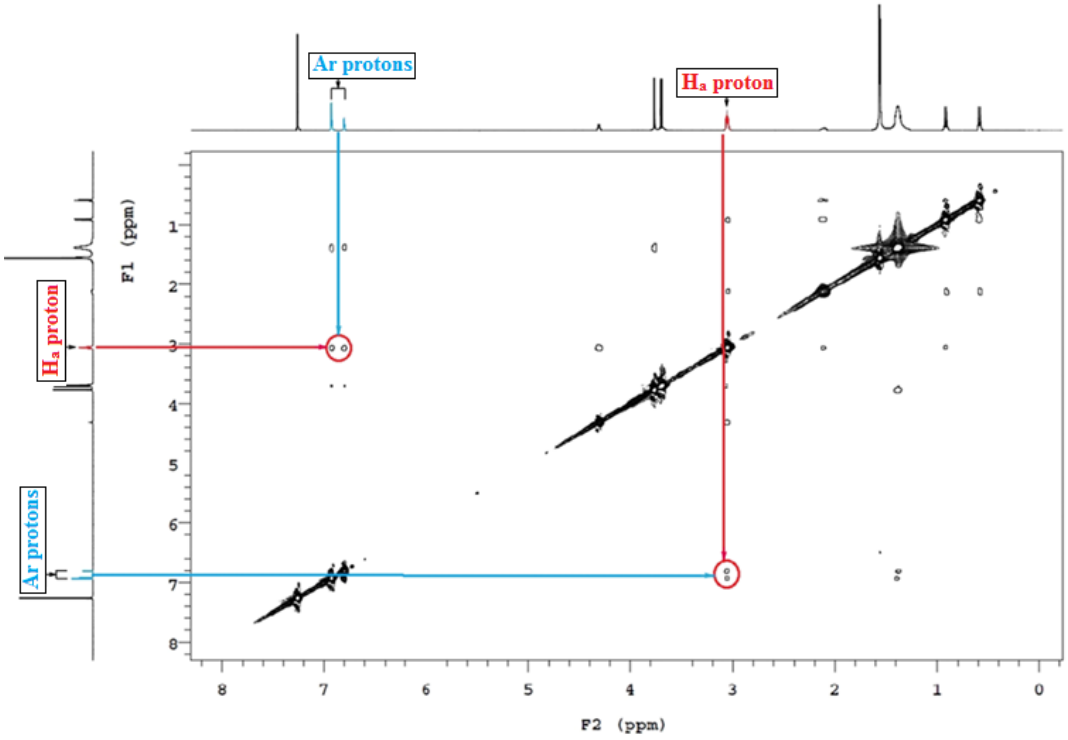


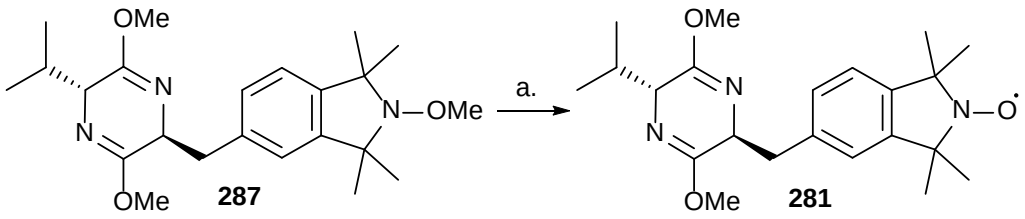
Figure 4.5. ROESY spectrum of **287** (400 MHz at 300 ms spin lock): showing the correlation between dihydropyrazine C_5 proton (red) and isoindoline aromatic protons (blue).

The strong C=N stretch of the dihydropyrazine ring was observed at 1695 cm^{-1} in the FTIR spectrum of **287**. The presence of the two C=N group of the dihydropyrazine ring was also confirmed by the carbon signals at 162 and 164 ppm in the ^{13}C NMR spectrum of **287**. In addition the molecular ion $[M + H]^+$ was observed at m/z 402 in the HRMS spectrum of **287**. Compound **287** was assessed to be >95% pure by HPLC with a melting range of 62-63 ^{o}C.

Having confirmed that compound **287** was a single diastereomer (2*S*, 5*R*), the next step was to deprotect the nitroxide moiety.

4.2.1.3 Synthesis of Dihydropyrazine TMIO 281 via Oxidation of the Methoxyamine Derivative 287

Oxidation of dihydropyrazine methoxyamine **287** to the corresponding nitroxide **181** was achieved under mild *m*CPBA oxidation conditions (**Scheme 4.6**) in the presence of sodium bicarbonate. To avoid a potential acid hydrolysis of the dihydropyrazine rings of **281** and **287**, sodium bicarbonate was employed to neutralize the *m*-chlorobenzoic acid by-product of the *m*CPBA reagent. The deprotection proceeded smoothly to furnish compound **281** as a yellow solid in high yield (81%) after purification by silica gel column chromatography.



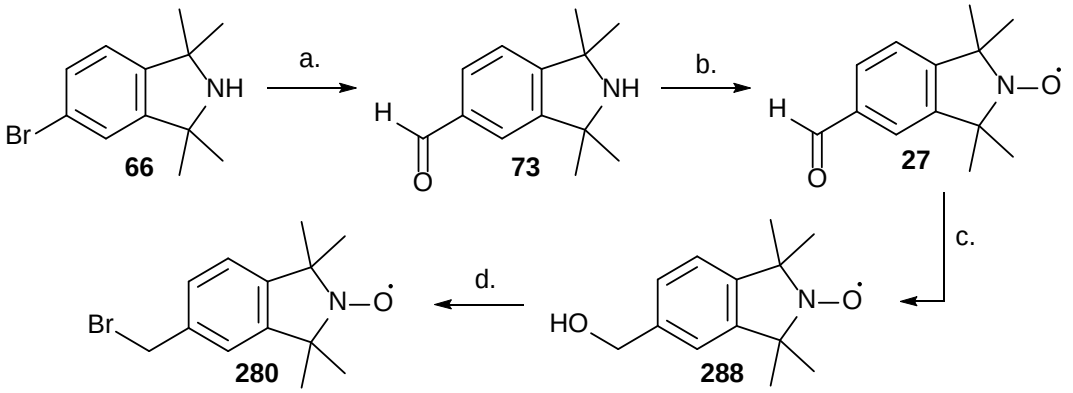
Scheme 4.6. Synthesis of dihydropyrazine nitroxide **281** via *m*CPBA demethylation of *N*-methoxyamine dihydropyrazine **287**. Reagents and conditions: a. *m*CPBA, $NaHCO_3$, DCM, 0 ^oC, 3 h, 81%.

As expected, a paramagnetic broadening effect of the nitroxide moiety was observed in the ^1H NMR spectrum of **281**. Despite that, however, each dihydropyrazine ring proton signal was conveniently observed and properly assigned. The isopropyl group protons were observed as broad singlets at 0.61 (CH_3), 0.85 (CH_3) and 2.14 (CH) ppm. The C_2, C_5 and the dimethoxy protons were also observed at 3.19 (1 H), 3.5 (1 H), and 3.72 (6 H) respectively. A diagnostic molecular ion plus sodium $[M + Na]^+$ was observed at m/z 409 in the HRMS spectrum of **281**. The purity of **281** was assessed by HPLC to be >95% and its measured melting range was 85-86 °C.

Having successfully employed (diastereoselectively) the Schöllkopf's approach in the alkylation of bis-lactim ether **275** with the benzyl bromide methoxyamine **283**, the next section explores the use of similar conditions for the alkylation of bis-lactim ether **275** with benzyl bromide nitroxide **280** as proposed earlier in **Scheme 4.3**.

4.2.1.4 Synthesis of Dihydropyrazine TMIO 181 via Benzyl Bromide TMIO 280

As mentioned earlier, benzyl bromide methoxyamine **283** was used in the synthesis of the dihydropyrazine nitroxide **181** to conveniently assess, by ^{1}H NMR spectroscopy, the enantioselectivity of the alkylation step of the Schöllkopf methodology. Having established the diastereoselectivity of the Schöllkopf's alkylation step, a similar reaction was also attempted with benzylic nitroxide **280** in place of methoxyamine **283**. As outlined in **Scheme 4.7** below, the benzylic nitroxide **280** was obtained in 4 steps from the bromoamine precursor **66**.

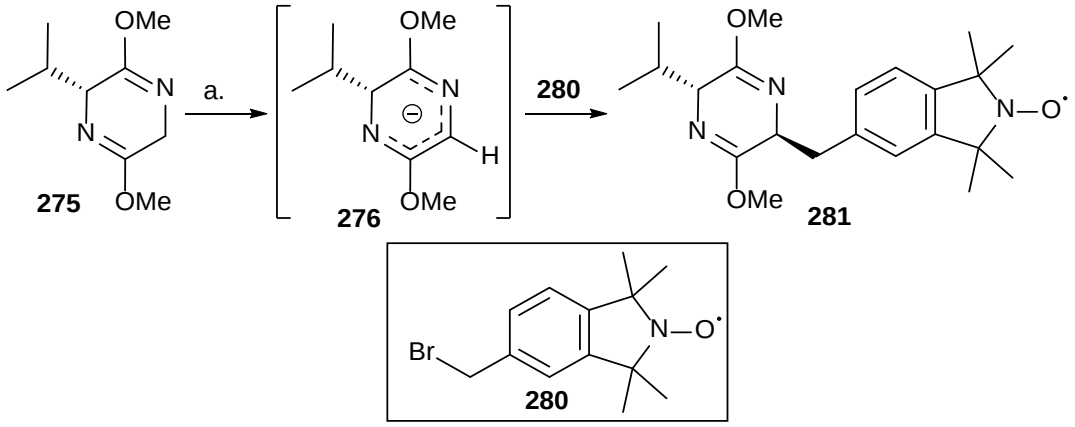


Scheme 4.7. Synthesis of benzyl bromide TMIO **280**. Reagents and conditions: a. *n*BuLi, THF, -78 ^{o}C, 15 min, then DMF, 3 h, 100%; b. *m*CPBA, DCM, 0 ^{o}C-RT, 2 h, 92%; c. $NaBH_4$, MeOH, 0 ^{o}C, 1.5 h, 86%; d. MsCl, TEA, 0 ^{o}C, 2 h, THF, Ar, then LiBr, Me_2CO, reflux, 2 h, 75%.

As detailed in **Chapter 3**, the formyl nitroxide **27** was obtained in two steps from the bromoamine **66**. First, the dianion generated from the lithaiation of **66** was quenched with DMF to quantitatively furnish 5-formyl amino derivative **73**. This was followed by a mild *m*CPBA oxidation of **73** to give the formyl nitroxide **27**. Subsequent reduction of compound **27** to the corresponding benzyl alcohol **288** was facilitated by sodium borohydride in one hour. The benzyl alcohol **288** was obtained in 86% yield after purification by column chromatography and recrystallization from cyclohexane. In the FTIR spectrum of **288**, the presence of a strong-broad OH absorption at 3425 cm^{-1} and the absence of a strong formyl C=O stretch around 1600-1700 cm^{-1} supported the transformation. Compound **288** was determined to be >95% pure by HPLC.

Like the methoxyamine counterpart **283**, the benzyl bromide nitroxide **280** was obtained by reacting benzyl alcohol **288** with methanesulfonyl chloride in the presence of triethylamine to produce a mesylate derivative. The mesyl group was then readily displaced *in situ* by bromide ions by reacting with lithium bromide to furnish the desired benzyl bromide derivative **280**. Compound **280** was characterized by HRMS and FTIR spectroscopy and its purity was assessed by HPLC (>95%).

Following similar alkylation conditions as described in **Scheme 4.5** above, the bis-lactim ether chiral auxiliary **275** was first lithiated with *n*-butyllithium and the resulting anion **276** was then reacted with benzyl bromide nitroxide **280** to furnish the dihydropyrazine nitroxide **281** in 78% yield (**Scheme 4.8**). To avoid a potential reaction of the bromide or the nitroxide moiety of **280** with *n*-butyllithium, a slight excess of the chiral auxiliary **275** was used.



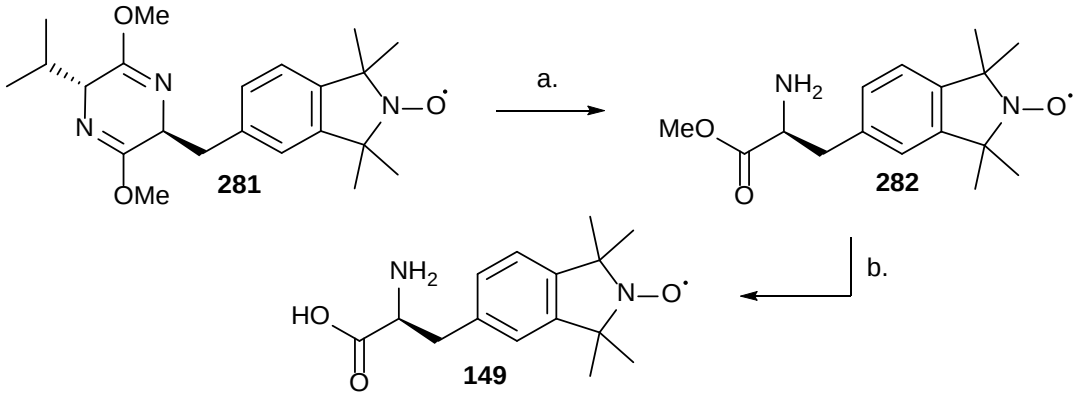
Scheme 4.8. Synthesis of dihydropyrazine nitroxide **281** via alkylation of bis-lactim ether **275** with benzyl bromide TMIO **280**. Reagents and conditions: a. *n*BuLi, -78 ^{o}C, 15 min, THF, then **280**, 3 h, 78%.

All characterization data obtained for dihydropyrazine **281** from the benzyl bromide nitroxide **280** route were consistent with those obtained from the benzyl bromide methoxyamine **283** route (**Scheme 4.5** and **Scheme 4.6**).

Although both routes give comparatively good yields, compound **281** was obtained via the benzyl bromide nitroxide **180** route in five steps from bromoamine precursor **66** while that of the methoxyamine **283** route was seven steps.

4.2.1.5 Hydrolysis of Dihydropyrazine 281 to Furnish L-Dopa-Nitroxide TMIO 149

A mild acid hydrolysis of the dihydropyrazine nitroxide **281** furnished the crude amino methyl ester **282** as a mixture with the D-valine methyl ester **279** (**Scheme 4.9**). Purification of the crude product mixture by silica gel column chromatography completely removed the D-valine methyl ester **279** side product. Compound **282** was obtained as a yellow solid (76%) and was determined to be >95% pure by HPLC.



Scheme 4.9. Hydrolysis of dihydropyrazine nitroxide **281** to the target L-Dopa TMIO **149**. Reagents and conditions: a. HCl, THF/H_2O, 3 h, 76%; b. LiOH, THF/H_2O, 2 h, 89%.

The characteristic primary amine (NH_2) stretching frequency was observed as two weak bands at 3382 and 3319 cm^{-1} in the FTIR spectrum of **282**. In addition, the strong C=O absorption observed at 1730 cm^{-1} confirmed the presence of methyl ester carbonyl group in **282**. The identity of **282** was further confirmed by the presence of the diagnostic $[M + H]^+$ and $[M + Na]^+$ ions at m/z 292 and 314 respectively in the HRMS spectrum. The significant paramagnetic broadening effects of the nitroxide moiety limited the characterization of **282** by ^1H NMR spectroscopy.

The target L-Dopa nitroxide **149** was obtained when a solution of the amino methyl ester **282** and three molar equivalents of lithium hydroxide were stirred at room temperature for 2 h. The amino acid **149** was obtained as a yellow precipitate at pH 7 in 89% yield. The identity of compound **149** was confirmed by the presence of the characteristic $[M + Na]^+$ ion at m/z 300 in the HRMS spectrum. A characteristic

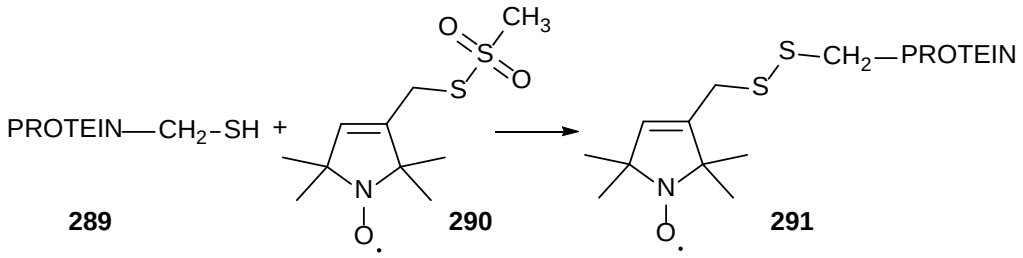
carboxylic acid O-H stretch was also observed at 3376 cm^{-1} in the FTIR spectrum of **149**. Compound **149** decomposed above 121 ^{o}C.

Having successfully employed the Schöllkopf methodology to synthesize the L-Dopa nitroxide analogue **149**, the approach was further employed in the synthesis of the novel rigid alkyne-linked α-amino acid nitroxide **151** as discussed in the next section. Compound **151** was designed as a potential site directed spin label for macromolecules such as peptides and proteins.

4.2.2 Schöllkopf Synthesis of the Rigid Alkyne-linked α-Amino Acid Nitroxide 151 as a Potential SDSL for Peptides and Proteins

Site-directed spin labelling (SDSL) is an effective technique used in combination with EPR spectroscopy to study the structure and conformational dynamics of macromolecules such as proteins, nucleic acids and carbohydrates.[57-62] The technique involves covalent attachment of a spin label to a site of interest on a target biomolecule. This enables structural and dynamic information to be obtained under conditions close to the physiological or actual functional state of the system under investigation.

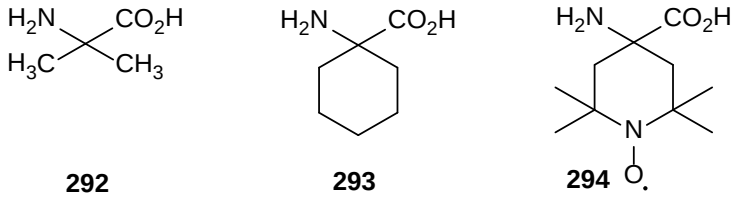
For structural elucidation of peptides and proteins, the methanethiosulfonate spin label **290** (MTSL) is the most widely used nitroxide compound in SDSL experiments.[63] In general, the protocol involves incorporating unique cysteine residues at selected sites of interest within the protein under study by cysteine substitution mutagenesis (**Scheme 4.10**). This is performed with minimal functional alterations to the protein.[64]



Scheme 4.10. Disulfide-linked MTSL-protein complex formation for SDSL studies.

Under appropriate conditions, sulfhydryl groups of mutated cysteine residues can react with MTSL **290** to form the disulfide-linked MTSL-protein complex **291**.[65] Despite the extensive use of the MTSL in SDSL-EPR experiments, the approach suffers from some notable drawbacks.[66] Firstly, the newly formed disulfide bond can be easily cleaved in the presence of a range of reducing agents such as dithiothreitol (DTT). Also, in some proteins, native cysteine residues are essential for their optimum structural and functional dynamics. Therefore modification of such cysteine residues with the MTSL to form a disulfide may not provide accurate structural and dynamic information regarding the native protein under study. Furthermore, the rotational flexibility along the five single bonds (between the pyrroline ring of the MTSL and the α-carbon of the protein backbone) often leads to increasing mobility of the spin label.[67] This consequently makes the position of the spin label more difficult to define. The rotation freedom along the five single bonds can also lead to isomerization about the disulfide bond. This in turn leads multiple undesirable motional components (such as decoupling of the motion of the spin label and protein) in the EPR spectra.

The most common alternative strategies to the MTSL that are used to introduce nitroxide spin labels within macromolecules are solid-phase peptide synthesis and click chemistry.[57,68-71] The first and the most widely used α-amino acid spin probe incorporated in a peptide via a peptide bond (solid phase peptide synthesis) is the paramagnetic 2,2,6,6-tetramethyl-*N*-oxyl-4-amino-4-carboxylic acid or TOAC **294** (**Figure 4.6**).[72-74]



292 **293** **294**

Figure 4.6. Common C^{α}-tetrasubstituted α-amino acids: Aminoisobutyric acid (Aib) **292**, 1-aminocyclohexane-1-carboxylic acid **293** (Ac6c) and TOAC **294**.

TOAC-coupled peptides show significant conformational restrictions.[75,76] This reduces the number of backbone conformations and rotational mobility. Most TOAC

spin label experiments give good structural information. However, the achiral nature of TOAC spin means other TOAC conformations do exist.[77,78] This may affect the secondary structure of peptides and alter spatial intermolecular and/or intramolecular distance measurements between two TOAC paramagnetic centres.[79] Thus, the aim of this work was to develop a new spin label that could minimize the drawbacks associated with rotational mobility, isomerization, multiple conformers, and achiral effects.

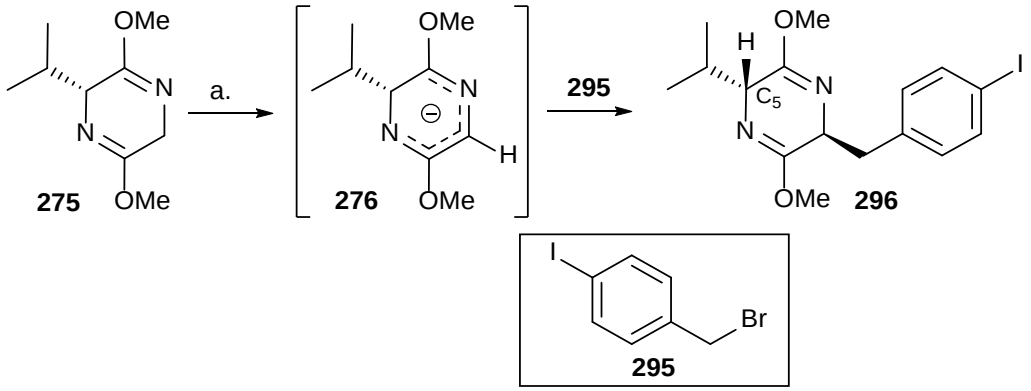
The target novel-rigid-chiral α-amino acid nitroxide spin label **151** could accurately provide relevant structural and dynamic information about a peptide under study. The design of this spin label is based on the more rigid isoindoline nitroxide ring class bridged to an L-phenylalanine α-amino acid by an alkyne linker. Due to its rigidity and chirality, biomolecular structural information could be obtained with compound **151** with minimal drawbacks associated with rotational mobility, isomerization, multiple conformers and achiral effects. Compound **151** could also be used to monitor cellular ROS-induced oxidative stress damage of peptides and proteins. Due to its expected high fluorescent quenching potential, **151** could also be used as a fluorescent probe to deduce more structural information of peptides and proteins. Under appropriate conditions, fluorescence would be restored when **151** is oxidized or reduced to its oxoammonium ion or hydroxylamine derivatives respectively.

The synthesis of compound **151** was carried out using the Schöllkopf's bis-lactim ether approach and palladium-catalyzed Sonogashira coupling reactions as discussed below.[45,46]

4.2.2.1 Alkylation of Schöllkopf's Bis-lactim Ether 275

As outlined in **Scheme 4.11** below, the first step towards compound **151** involved lithiation of the bis-lactim ether (2*R*)-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine **275** with *n*-butyllithium, followed by the reaction of the resulting enolate **276** with 4-iodobenzyl bromide **295** to furnish the alkylated dihydropyrazine **296** in 86% yield. Compound **296** was obtained in its pure form as a single diasteromer and its identity was confirmed by NMR spectroscopy, HRMS and FTIR analysis. Like **287**, compound **296** also adopts an "aryl-inside" conformation. This

was verified by the difference in the chemical shift for the C_5 proton of **296** (observed at 3.4 ppm) and the starting material **275** (observed at 4 ppm)- an upfield shift of 0.6 ppm. Further confirmation was obtained by the spatial correlation between the C_5 proton and the para-substituted aromatic ring protons in the ROESY spectrum of **296**.

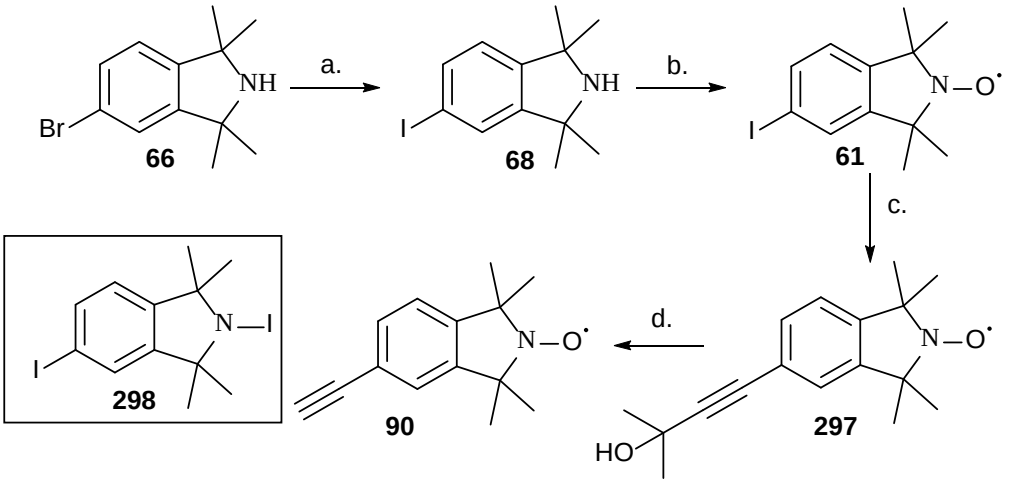


Scheme 4.11. Alkylation of bis-lactim ether **275** with 4-iodobenzyl bromide **295**. Reagents and conditions: a. *n*BuLi, THF, -78^{o}C, 15 min, then **295**, 4 h, 86%.

4.2.2.2 Synthesis of Alkyne TMIO 90

Following successful diastereoselective alkylation of Schöllkopf's chiral auxiliary **275**, the next step was to synthesize alkyne TMIO **90** by following previous literature procedures as outlined in **Scheme 4.12** below.[80,81] The bromoamine starting material **66** was synthesized from commercially available phthalic anhydride **167** in 3 steps as described in **Chapter 2**. Lithiation of bromoamine **66** with *n*-butyllithium resulted into the formation of a dianion which, upon quenching with iodine, furnished the crude 2,5-diiodoisoindoline compound **298**. The diiodo compound **298** was then reduced by aqueous hydrogen peroxide solution in the presence of sodium bicarbonate to afford the 5-iodoisoindoline derivative **68**. The 5-iodo nitroxide **61** was obtained almost quantitatively by subjecting compound **68** to mild *m*CPBA oxidation conditions. Then a palladium-catalyzed copper-free Sonogashira coupling reaction of 5-iodo nitroxide **61** with a fivefold excess of 2-methyl-3-butyn-2-ol furnished the protected acetylene nitroxide **297** in 70% yield. Compound **297** was deprotected with potassium hydroxide in refluxing anhydrous toluene to give the desired alkyne TMIO **90** as a bright yellow solid in 93% yield. All characterization

data obtained for the synthesis of **90** were consistent with those reported in the literature.[80,81]

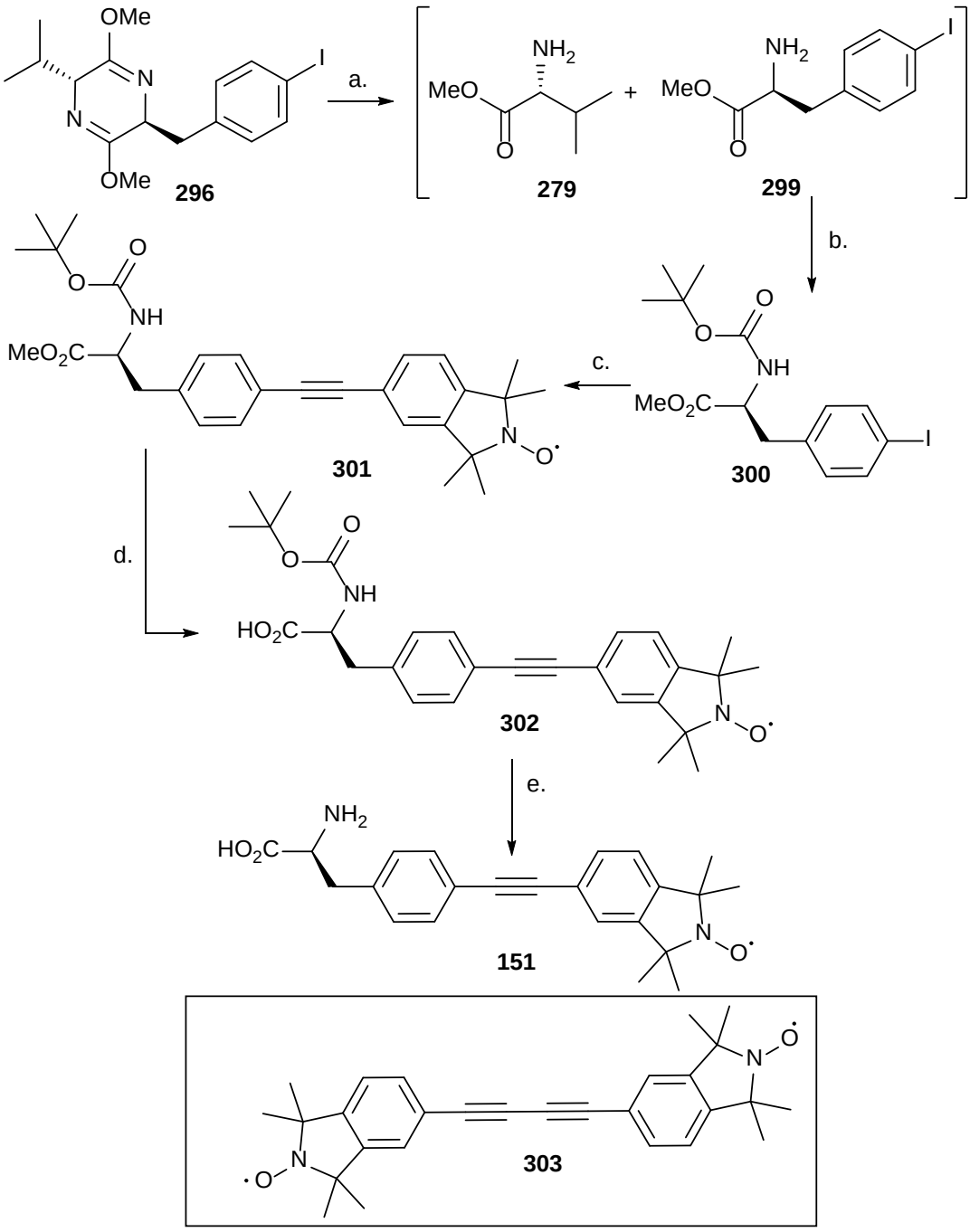


Scheme 4.12. Synthesis of alkyne TMIO **90**. Reagents and conditions: a. *n*BuLi, THF, -78°C, 10 min, then I_2, $NaHCO_3$, H_2O_2, 80%; b. *m*CPBA, DCM, 0 °C, 3 h, 87%; c. 2-methyl-3-butyn-2-ol, DABCO, $Pd(OAc)_2$, MeCN, Ar, reflux, 24 h, 70%; d. KOH, toluene, reflux, 30 min, 93%.

With the alkyne TMIO **90** and the 4-iodobenzyl dihydropyrazine **296** in hand, the synthesis of the target compound **151** from these two intermediates was accomplished via two routes as described below.

4.2.2.3 Synthesis of Alkyne-linked α-Amino Acid Nitroxide 151 via Sonogashira Coupling

In the first route (**Scheme 4.13**), 4-iodobenzyl dihydropyrazine **296** was first hydrolyzed under acidic conditions to give an equimolar mixture of the (*S*)-4-iodophenylalanine **291** and D-valine **279** methyl esters as verified by ^{1}H NMR spectroscopy. Both amino esters were then protected with Boc groups by allowing the crude mixture to react with Boc-anhydride in the presence of triethylamine. Purification of the crude product by silica gel chromatography afforded (*S*)-(*N*-*t*-butoxycarbonyl)-4-iodophenylalanine methyl ester **300** as a white solid in 66% yield (over the two steps from **296**). The *t*-butoxy protons of the Boc-protecting group were shown as a singlet at 1.42 ppm that integrated for 9 protons in the ^{1}H NMR spectrum of **300**.



Scheme 4.13. Synthesis of the rigid alkyne-linked α-amino acid SDSL **151** via Sonogashira coupling of Boc-protected phenylalanine methyl ester **300** with alkyne TMIO **90**. Reagents and conditions: a. TFA, MeCN/H_2O, 3 h; b. $(Boc)_2O$, TEA, RT, O/N, (66% from **296**); c. **90**, $PdCl_2(PPh_3)_2$, CuI, TEA, RT, O/N, 66%; d. LiOH, H_2O/THF, 2 h, 93%; e. HCl, dioxane/H_2O, O/N, 96%.

Also, the methylene (CH_2) group protons were observed as two doublet of doublets at 2.95 and 3 ppm. The singlet at 3.72 ppm (3 protons) and the broad doublet at 4.56

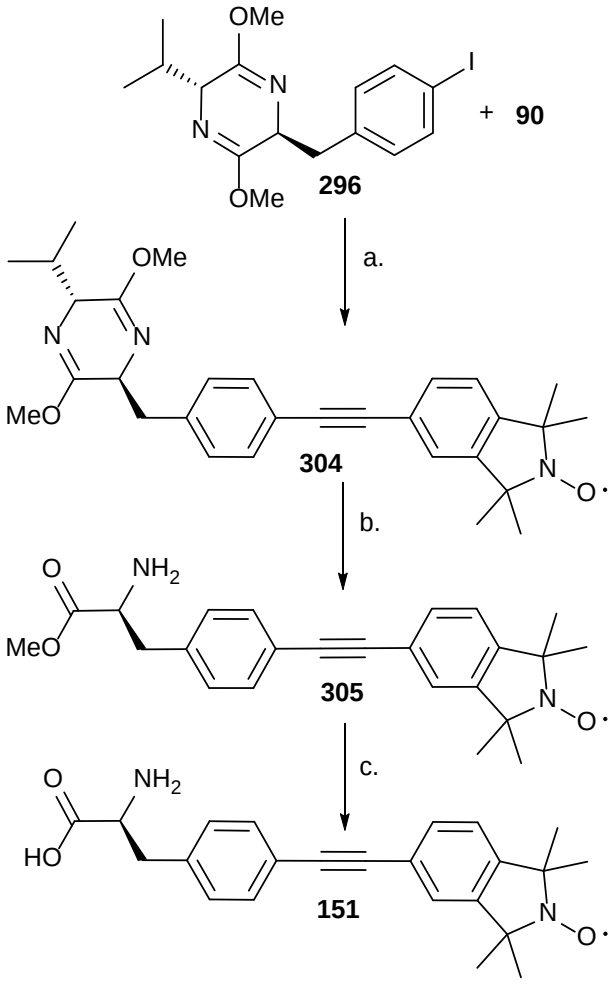
ppm (1 proton) were assigned to the methoxy protons of the methyl ester group and C_2 (α-carbon CH) proton respectively. The two doublets in the aromatic region (at 6.78 and 7.61 ppm), each integrating for 2 protons, were assigned to the para-substituted phenyl ring protons. Further evidence to support the identity of **300** was obtained from the presence of two strong C=O stretching frequencies observed at 1735 and 1684 cm^{-1} in the FTIR spectrum of **300**. This was consistent with the presence of the methyl ester and the Boc-protecting group. A medium NH stretch (of the Boc-NH group) was also observed at 3343 cm^{-1}. Other characterization data obtained to further support the identity of **300** included ^{13}C NMR spectroscopy and HRMS analysis. Compound **300** was determined to be >95% pure by HPLC analysis.

Bis(triphenylphosphine)palladium (II) dichloride facilitated Sonogashira reaction of **300** with the alkyne TMIO **90** in the presence of triethylamine and catalytic copper (I) iodide afforded the rigid alkyne TMIO-linked Boc-protected phenylalanine methyl ester **301**. Although the coupling proceeded smoothly, it was however accompanied by the formation of the homodimer alkyne TMIO side product **303** as verified by HRMS analysis. Hence the alkyne TMIO **90** was used in slight excess. Compound **301** was obtained in 66% yield as a yellow solid after the crude residue was subjected to flash column chromatography. The identity of compound **301** was confirmed by the presence of the characteristic $[M + H]^+$, $[M + 2H]^+$ and $[M + Na]^+$ ions at m/z 492, 493 and 514 respectively in the HRMS spectrum of **301**. Despite the paramagnetic broadening effect of the nitroxide moiety, the *N*-Boc phenylalanine methyl ester fragment protons were assigned in the ^1H NMR spectrum of **301**. Compound **301** was assessed to be >95% pure by HPLC and its melting range was determined to be 183-184 ^oC.

The methyl ester group of **301** was then hydrolyzed with lithium hydroxide to give the corresponding carboxylic acid **302** in 93% yield. Although the carboxylic acid proton could not be assigned, the loss of the methoxy protons signal around 3.7 ppm in the ^1H NMR spectrum of **302** evidently supported the successful transformation. Furthermore, a characteristic broad O-H stretching absorption of the carboxylic acid group was observed in the FTIR spectrum of **302** (ranging from 2500 to 3500 cm^{-1}). The HRMS spectrum of **302** showed the distinct $[M + H]^+$ mass at m/z 462. The

purity of **302** was confirmed by HPLC analysis to be >95%. The Boc-protecting group of **302** was then removed under mild hydrochloric acid conditions to furnish the target alkyne-linked α-amino acid nitroxide **151** as a yellow precipitate in 96% yield. HRMS analysis confirmed the identity of target compound **151** by the presence of the diagnostic $[M + 2H]^+$ ion at m/z 379.

Alternatively, the target compound **151** was obtained by first subjecting the 4-iodobenzyl dihydropyrazine **296** and the alkyne TMIO **90** to similar Sonogashira coupling conditions (used in the synthesis of **301** above) prior to hydrolysis of the dihydropyrazine ring (**Scheme 4.14**).



Scheme 4.14. Synthesis of the rigid alkyne-linked α-amino acid SDSL **151** via Sonogashira coupling of dihydropyrazine **296** with the alkyne TMIO **90**. Reagents and conditions: a. $PdCl_2(PPh_3)_2$, CuI, TEA, RT, O/N, 73%; b. HCl, MeCN, 2 h, 93%; c. LiOH, H_2O/THF, 2 h, 86%.

The alkyne-linked dihydropyrazine **304** was obtained as a yellow solid in 73% yield after purification by silica gel chromatography. Evidence to support the synthesis of **304** was obtained from HRMS, FTIR and ^{1}H NMR spectroscopy. Despite the paramagnetic broadening effect of the nitroxide moiety, each proton of the benzyl dihydropyrazine fragment was correctly assigned in the ^{1}H NMR spectrum of **304**. Protons of the isopropyl group were observed as broad doublets at 0.62 (CH_3), 0.96 (CH_3) and 2.17 (CH) ppm. The C_5 and C_2 protons were also observed as broad singlets at 3.38 (1 H) and 4.34 (1 H) ppm respectivel. The two singlets at 3.69 and 3.73 ppm (each integrated for 3 H) were assigned to the two methoxy groups of the dihydropyrazine ring. The broad doublets at 7.11 and 7.37, each integrated for 2 protons, were assigned to the para-substituted phenyl ring protons. In the FTIR spectrum of **304**, the C=N stretch of the dihydropyrazine ring was observed as a strong band at 1693 cm^{-1}. The HRMS spectrum of **304** further confirmed its identity by the presence of the $[M + H]^+$ and $[M + Na]^+$ masses at m/z 487 and 509 respectively. The purity of compound **304** was assessed by HPLC to be >95% and its measured melting range was 85-86 °C.

Hydrolysis of the dihydropyrazine ring of **304** with dilute hydrochloric acid furnished the amino methyl ester **305** in 99% yield. The two medium absorptions at 3309 and 3369 cm^{-1} in the FTIR spectrum of **305** are the characteristic NH stretch of the primary amino group. In addition, a strong C=O stretches of the methyl ester was also observed at 1741 cm^{-1}. The protons of the phenylalanine methyl ester fragment were accounted for in the ^{1}H NMR spectrum of **305**. Further evidence to support the identity of **305** was obtained from HRMS analysis. The purity of compound **305** was assessed by HPLC to be >95%.

The target alkyne-linked α-amino acid nitroxide **151** was obtained almost quantitatively when the phenylalanine methyl ester **305** was reacted with three molar equivalents of aqueous lithium hydroxide at room temperature. All characterization data obtained for **151** via this route matched that obtained by the first route. Although each of the two routes furnished the target compound **151** in comparatively good yields, the second route (**Scheme 4.14**) had two fewer steps than the first (**Scheme 4.13**). The overall yield for routes 1 and 2 were 60 and 64% respectively. Compound **151** will be assessed for its potential spin label application in peptides and proteins.

4.2.3 The Attempted Synthesis of Ritalin-TMIO Analogue 150

Methylphenedate **148** (MPH, Ritalin) is the most common medication for the treatment of ADHD. Structurally, MPH has four stereoisomers due to the presence of two stereocentres (**Figure 4.7**). While the threo-isomers possess significant therapeutic effect, the erythro counterparts display very little activity.[82] The toxic hypertensive side effects of MPH are known to arise mainly from the use of the erythro isomers. Although the D-threo isomer is up to 38 times more potent than the L-threo counterpart, Ritalin is currently administered as a racemic mixture of both threo isomers.[83-85] Despite such significant difference in therapeutic efficacy, there is currently not many reported enantioselective synthesis of the D-threo isomer in the literature.

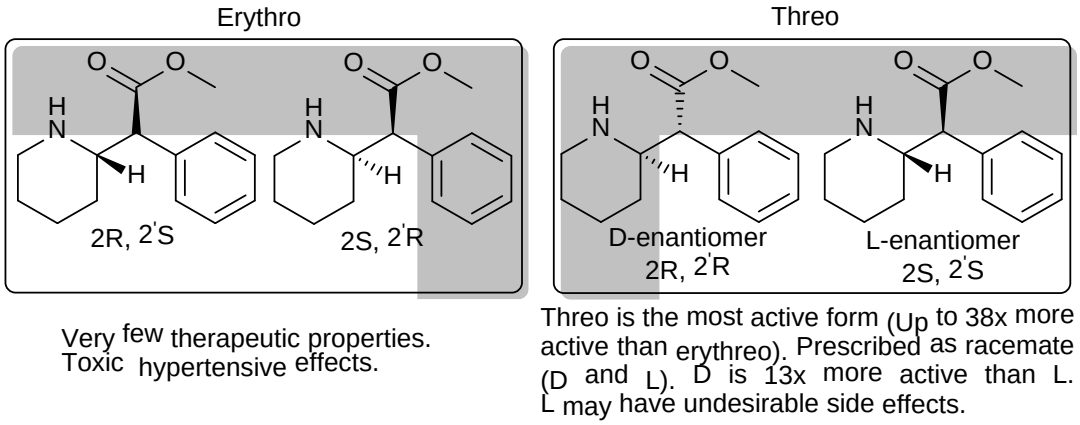
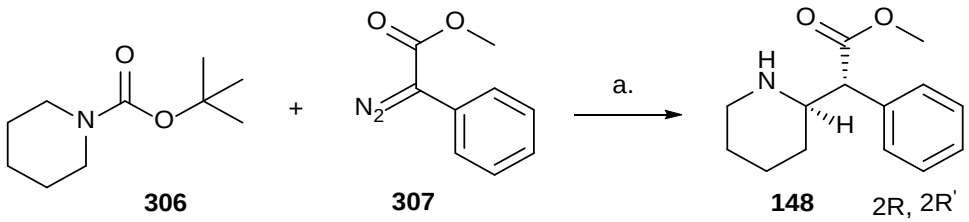


Figure 4.7. Erythro and threo isomers of methylphenedate (MPH).

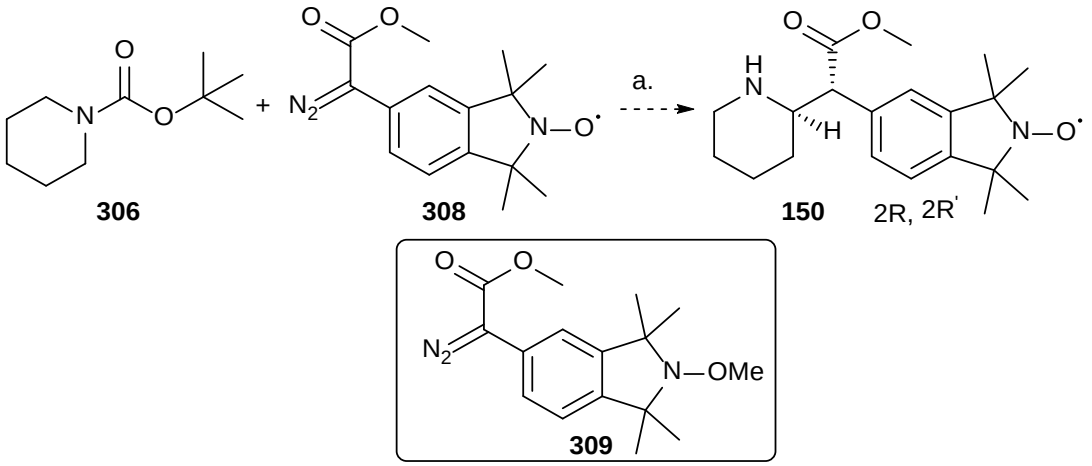
The synthesis of the target nitroxide-Ritalin derivative described here is based on the highly regio-, diastereoselective rhodium-catalyzed intermolecular C-H insertion approach that employs metal cabenoid intermediates.[86-88] Developed by Davies and coworkers, the approach uses a dirhodium catalyst such as Rh_2(S-DOSP)$_2$ to insert methyl phenyldiazoacetate **307** into *N*-Boc-piperidine **306** to give the D-threo-methylephenedate **148** in 86% diastereomeric excess (**Scheme 4.15**). The mechanism for this reaction is given in the review article by Davies and coworkers.[88]

A similar approach was attempted for the synthesis of the target Ritalin nitroxide analogue **150** as outlined in **Scheme 4.16** below. However, to determine the diastereoselectivity of this method by ^{1}H NMR spectroscopy, the dirhodium-

catalyzed intermolecular C-H insertion was first attempted on the methoxyamine derivative **309** of methyl diazoacetate TMIO **308**. Since the required *N*-Boc-piperidine **306** and diazoacetate methoxyamine **309** starting materials were not readily available, their syntheses are discussed below.



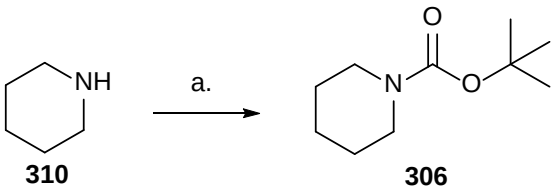
Scheme 4.15. Dirhodium-catalyzed asymmetric synthesis of Ritalin **148**. Reagents and conditions: a. Rh_2(S-DOSP)$_2$, hexane, -50 ^oC; then TFA.



Scheme 4.16. Proposed asymmetric synthesis of **150** via dirhodium-catalyzed intermolecular C-H insertion of methyl aryldiazoacetate **308**. Reagents and conditions: a. Rh_2(S-DOSP)$_2$, hexane, -50 ^oC; then TFA.

4.2.3.1 Synthesis of *N*-Boc Piperidine 306

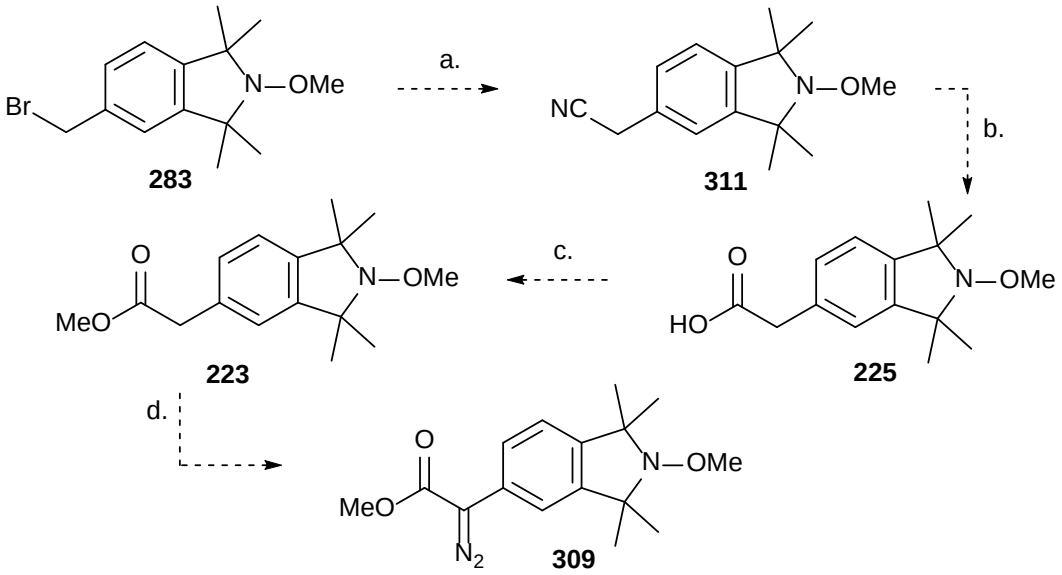
Piperidine **310** was readily converted to the *N*-Boc-protected derivative **306** by allowing it to react at room temperature with Boc-anhydride for two hours (**Scheme 4.17**). The crude product was purified by Kugelrohr distillation to give compound **306** as clear oil in 95% yield. All characterization data obtained for compound **306** were in agreement with the literature.[89]



Scheme 4.17. Synthesis of *N*-Boc piperidine **306**. Reagents and conditions: a. Boc_2O, 0 ^oC-RT, THF, 2 h, 95%.

4.2.3.2 Synthesis of Methyl Diazoacetate Methoxyamine 309

Scheme 4.18 below depicts the proposed route for the synthesis of diazoacetate **309** from the benzyl bromide intermediate **283**. Compound **283** was synthesized as per **Scheme 4.4**.

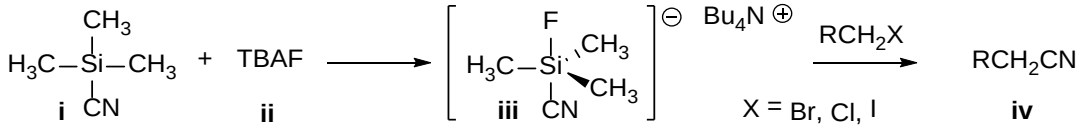


Scheme 4.18. Synthesis of methyl diazoacetate methoxyamine **309**. Reagents and conditions: a. TMSCN, TBAF, MeCN, reflux; b. 5 M KOH, reflux; c. MeOH, H_2SO_4 (98%), reflux; d. DBU, *p*-ABSA, MeCN, RT, Ar.

4.2.3.3 Synthesis of Benzylcyanide Methoxyamine 311 via Cyanation of 283

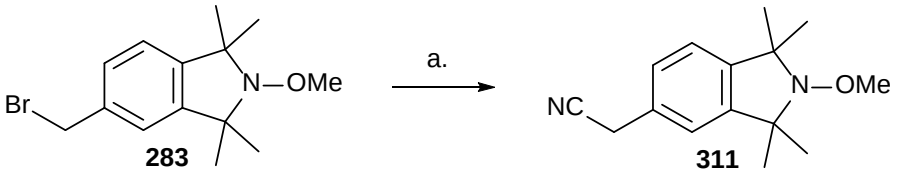
Cyanation of benzyl bromide **283** was facilitated by trimethylsilyl cyanide (TMSCN) as the cyanide source. TMSCN is a stabilized hydrogen cyanide that is commonly employed (in the place of NaCN or KCN) for direct nucleophilic substitution of alkyl halides. In such cases, the cyanation reaction is usually carried out in the presence of

an additive such as a fluoride compound, Lewis acid or a metal oxide.[90-93] DeShong and coworkers employed tetrabutylammonium fluoride (TBAF) as an additive to successfully cyanate a range of benzylic halides and azides in excellent yield (**Scheme 4.19**).[93] In that study, TBAF (**ii**) was reported to form a hypervalent cyanosilicate intermediate (**iii**) with TMSCN (**i**) which in turn facilitates the substitution of benzyl halide substrates to the corresponding benzyl cyanides (**iv**).



Scheme 4.19. Proposed mechanism for the cyanation of benzylic halides with TMSCN in the presence of TBAF. Adapted from DeShong *et al.*[93]

Following a similar protocol, the benzyl bromide methoxyamine **283** was successfully converted to the corresponding benzyl cyanide **311** in 81% yield and was obtained as a white solid. The procedure involved refluxing a reaction mixture of benzyl bromide **283**, TMSCN and TBAF in anhydrous acetonitrile (**Scheme 4.20**). Complete conversion was observed (by TLC analysis) after three hours.

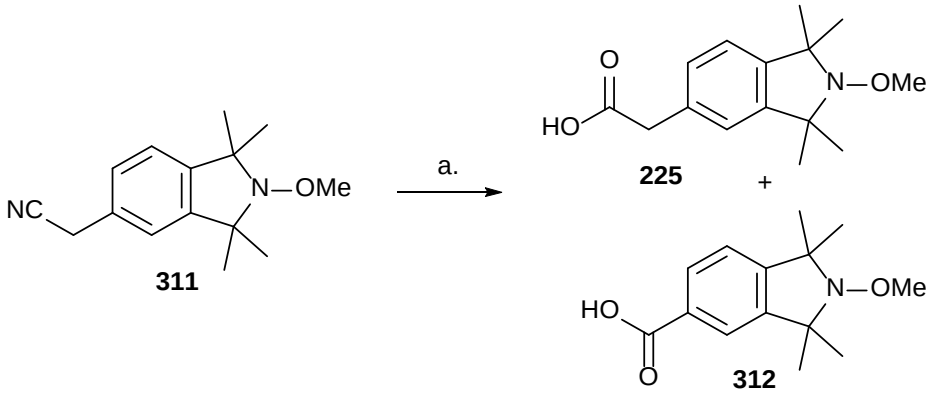


Scheme 4.20. Synthesis of benzyl cyanide methoxyamine **311** via TMSCN facilitated cyanation of benzylbromide methoxyamine **283**. Reagents and conditions: a. TMSCN, TBAF, MeCN, reflux, 3 h, 81%.

Evidence to support the transformation was obtained from the appearance of a characteristic C≡N stretch at 2250 cm^{-1} in the FTIR spectrum of **311**. In comparison to the benzyl bromide methoxyamine precursor **283**, an extra carbon peak was observed at 117.9 ppm in the ^{13}C NMR spectrum of **311**. This further supported the presence of a cyano group. Compound **311** was also characterized by HRMS and ^{1}H NMR spectroscopy and its purity was verified by HPLC analysis to be >95%. The melting range for **311** was determined to be 57-58 ^{o}C.

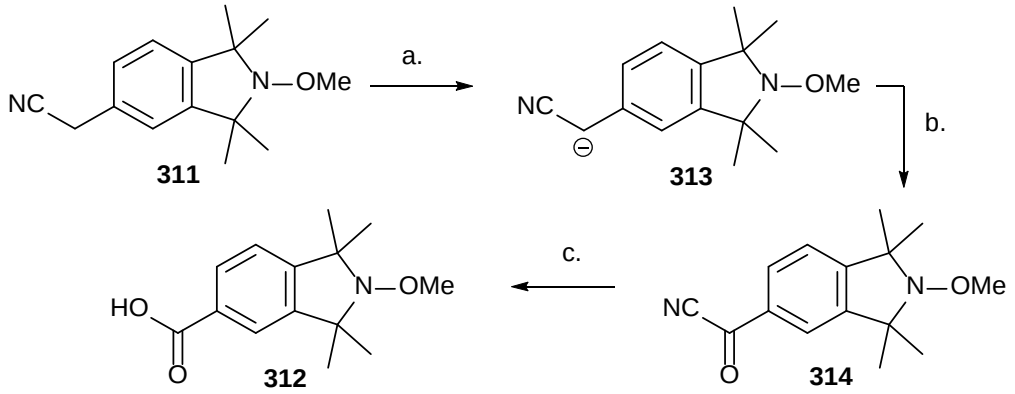
4.2.3.4 Synthesis of 2-Methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindoline 223 via Hydrolysis of Benzyl Cyanide 311

Nitriles are commonly hydrolyzed to the corresponding carboxylic acids under refluxing basic or acidic conditions. However, basic hydrolysis of **311** in refluxing potassium hydroxide solution (**Scheme 4.21**) furnished almost an equal mixture of the desired product **225** and, surprisingly, CTMIO methoxyamine derivative **312** as verified by the ^{1}H NMR and HRMS spectra of the crude product. A similar result was obtained with the benzyl cyanide nitroxide counterpart. Attempts to separate the crude mixture using conventional separation techniques (chromatography, trituration and recrystallization) were unsuccessful.



Scheme 4.21. Basic hydrolysis of benzyl cyanide *N*-methoxamine **311**. Reagents and conditions: a. 5 M, KOH, reflux, O/N.

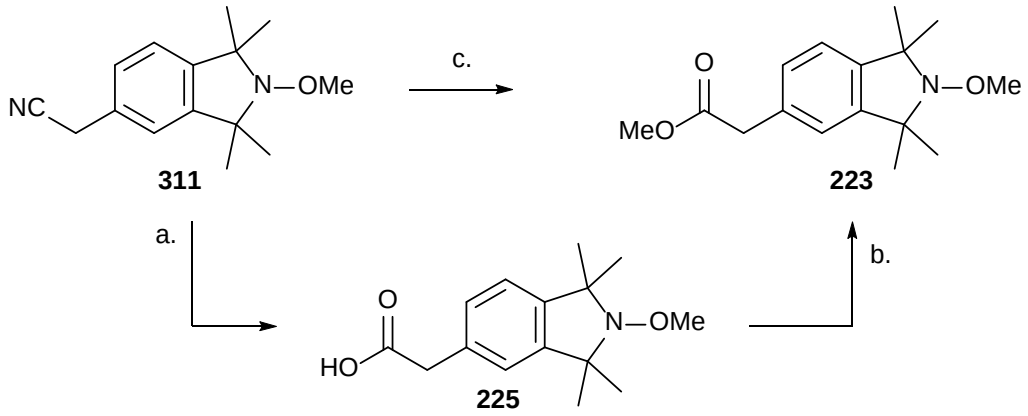
The unexpected formation of compound **312** under such basic hydrolysis conditions can be rationalized as follows (**Scheme 4.22**). The benzylic protons (CH_2) are the most acidic in the nitrile intermediate **311**. Gokel *et al.* proposed that the anion **313** generated by the deprotonation of the nitrile precursor **311** could likely be oxidized by molecular oxygen to give the acyl cyanide intermediate **314**.[94] The acyl cyanide intermediate **314** can then be readily cleaved by potassium hydroxide to give the CTMIO methoxyamine derivative **312**.



Scheme 4.22. Gokel *et al.* proposed mechanism for oxidative hydrolysis and cleavage of nitrile **311** to the carboxylic acid **312**.[94] Reagents and conditions: a. KOH; b. O_2; c. KOH, H_2O.

Similar oxidative hydrolysis (and cleavage) of nitriles are reported in the literature.[94,95] However, the nitrile methoxyamine **311** was successfully hydrolyzed exclusively to the desired carboxylic acid **225** (as verified by ^{1}H NMR spectroscopy and HRMS analysis) by refluxing in concentrated sulfuric acid solution (**Scheme 4.23**). The carboxylic acid **225** was obtained in as a white solid in 84% yield. The transformation was supported by the presence of a strong C=O and a broad OH absorptions at 1737 and 3382 cm^{-1} respectively in the FTIR spectrum of **225**. The mass of the molecular ion plus proton $[M + H]^+$ was observed at m/z 264 in the HRMS spectrum. Compound **225** was >94% pure by HPLC analysis and its melting range was determined to be 115-116 ^{o}C.

The methyl ester **223** was readily obtained by refluxing the carboxylic acid **225** in methanol in the presence of a catalytic amount of concentrated sulfuric acid. The methylene and the two methoxy protons were observed at 3.61, 3.69 and 3.77 ppm respectively in the ^{1}H NMR spectrum of **223**. Also, the loss of the carboxylic acid OH stretch in the FTIR spectrum of **223** further supported the successful esterification of **225**. Notably, the methyl ester **223** was also obtained directly from the benzyl cyanide **311**, in comparatively high yield (85%), when the acid hydrolysis was carried out in aqueous methanol (**Scheme 4.23.c**).



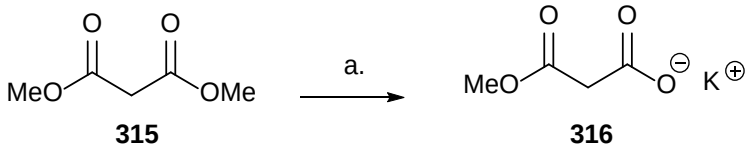
Scheme 4.23. Acid hydrolysis of benzyl cyanide *N*-methoxamine **302**. Reagents and conditions: a. H_2SO_4 (98%), reflux, O/N, 84%; b. MeOH, H_2SO_4 (98%), reflux, O/N, 87%; d. MeOH, H_2SO_4 (98%), reflux, O/N, 85%.

Although the methyl ester **223** was obtained from benzyl bromide precursor **283** in three steps, however, the total number of steps to **223** from the bromomethoxyamine **23** precursor was six. Based on a recent palladium-catalyzed decarboxylative coupling of aryl halides with malonates developed by Lui and coworkers,[96] the methyl ester **223** was, alternatively, readily obtained directly from the bromomethoxyamine **23** in one step. The protocol is discussed in the next section.

4.2.3.5 Synthesis of Methyl Ester 223 from Bromomethoxyamine 23

Alternatively, compound **223** was obtained directly from bromomethoxyamine **23** via a palladium-catalyzed decarboxylative coupling reported by Lui and coworkers.[96] In that study, a series of α-aryl nitriles were successfully synthesized in high yields from readily available cyanoacetate salts and appropriate aryl halides. The coupling was facilitated by allyl palladium chloride dimer and a phosphine ligand such as 2,2′-bis(diphenylphosphino)-1,1′-binaphthalene (BINAP) or 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl (S-Phos). To apply a similar decarboxylative coupling protocol in the synthesis of methyl ester **223** from the bromoamine precursor **23**, potassium malonate **316** was employed in the place of the cyanoacetate salts. First, the malonate monoester salt **316** was readily prepared by reacting equimolar amounts of methyl malonate **315** with potassium hydroxide in methanol (**Scheme 4.24**). The salt **316** was obtained quantitatively as a white powdery solid after washing the crude

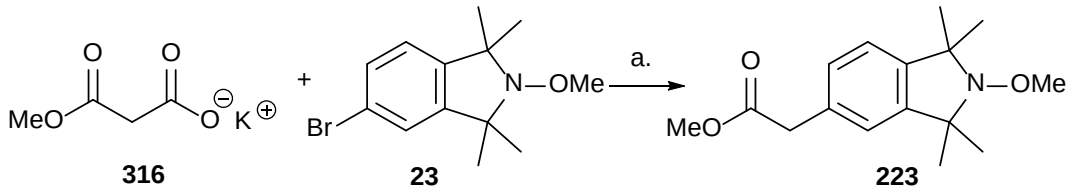
precipitate with diethyl ether and drying in a vacuum oven. All characterization data obtained for **316** were in agreement with the literature.[96]



315 **316**

Scheme 4.24. Synthesis of potassium malonate **316**. Reagents and conditions: a. KOH, MeOH, 0 ^{o}C-RT, O/N, 97%.

To access the methyl ester **223**, the oxidative decarboxylative coupling protocol involved refluxing a degassed reaction mixture of bromomethoxyamine **23** and malonate **316** in the presence of catalytic amounts of allyl palladium chloride dimer, BINAP, and DMAP (**Scheme 4.25**). Compound **223** was obtained in 54% after purification by silica gel column chromatography. All characterization data obtained herein were in agreement with those obtained via **Scheme 4.23** above.



316 **23** **223**

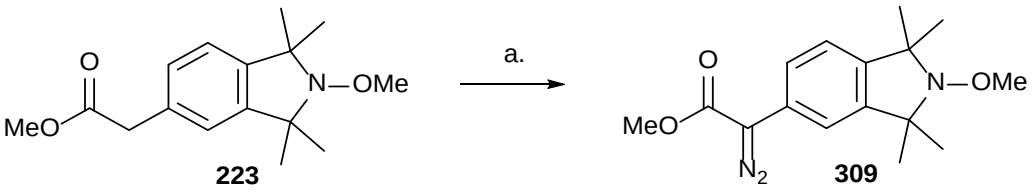
Scheme 4.25. Synthesis of methyl ester **223** via palladium-catalyzed oxidative-decarboxylative coupling of bromomethoxyamine **23** and potassium malonate **316**. Reagents and conditions: a. $[Pd(Allyl)Cl]_2$, BINAP, DMAP, mesitylene, reflux, Ar, 1d, 54%.

The methyl ester **223** was obtained in 54% via the single oxidative decarboxylative coupling step whereas in comparison the nitrile hydrolysis route (**Scheme 4.4**, **Scheme 4.20** and **Scheme 4.23**) gave an overall yield of 52% in five or six steps from **23**.

4.2.3.6 Synthesis of Methyl Diazoacetate Methoxyamine 309

α-Diazo carbonyl compounds are versatile building blocks widely employed in a variety of useful synthetic transformations such as the Wolff rearrangement, aromatic

cycloaddition, cyclization of unsaturated substrates and catalytic C-H insertion reactions.[97] Due to their structure and high nitrogen content, diazo compounds are highly reactive. Diazo compounds are thermally unstable and acid labile. Under most reaction conditions, they generally decompose to give the thermodynamically stable dinitrogen. One approach commonly used to access diazo carbonyl compounds is the Regitz diazo transfer reaction.[98] The approach generally involves a base-catalyzed reaction of *p*-toluenesulfonyl (tosyl) azide with an active methylene group (next to electron withdrawing groups). DBU is the most common base employed in the Regitz diazo transfer reaction. Despite the wide use of tosyl azide as the source of nitrogen in the diazo transfer reactions, the *p*-acetamidobenzenesulfonyl azide (*p*-ABSA) derivative has also been successfully employed.[99] As shown in **Scheme 4.26** below, *p*-ABSA was employed as the diazo transfer reagent in the synthesis of the diazo ester **309** from active methylene group of the methyl ester **223**.



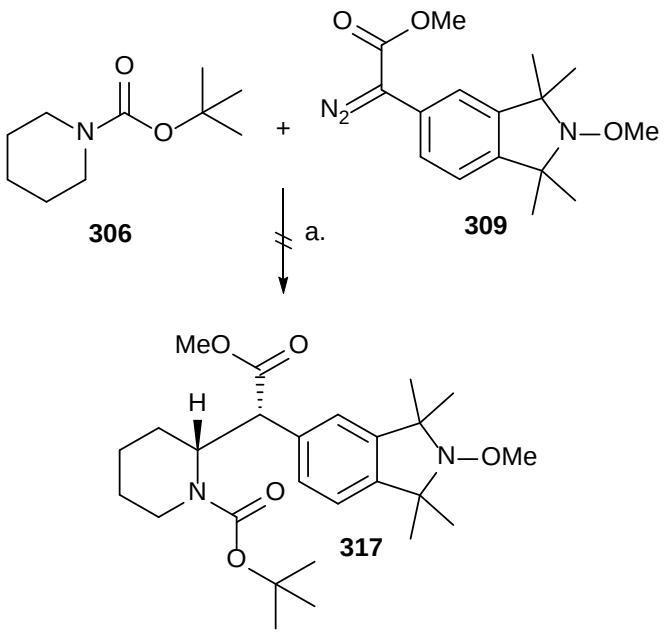
Scheme 4.26. Synthesis of methyl diazoacetate methoxyamine **309** via Regitz's diazo transfer reaction of methyl ester **223**. Reagents and conditions: a. *p*-ABSA, DBU, MeCN, RT, Ar, 1 d, 86%.

Thus, the α-diazo methyl ester **309** was prepared in excellent yield when compound **223** was allowed to react with *p*-ABSA in the presence of DBU for one day. Compound **309** was obtained as a bright yellow solid in 86% yield following purification by silica gel column chromatography. The characteristic 'diazo band' (CN≡N stretch) was observed as a strong signal at 2107 cm^{-1} in the FTIR spectrum of **300**. Also, the loss of the distinct methylene (CH_2) protons signal of **223** at 3.61 ppm in the ^1H NMR spectrum of the isolated product further supported the formation of the α-diazo methyl ester **309**. HRMS analysis of **309** confirmed the presence of the $[M + H]^+$ ion at m/z 304. Compound **309** was >95% pure by HPLC analysis and its melting range was determined to be 91-92 ^oC. Notably, the α-diazo methyl ester

309 was slightly unstable at room temperature and decomposed over time to the methyl ester **223**. This was verified by TLC and ^{1}H NMR spectroscopy analysis.

4.2.3.7 Attempted Synthesis of Methoxyamine Derivative of Ritalin-Nitroixide 150 via Dirhodium-Catalyzed Intermolecular C-H Insertion

As mentioned earlier, the key and ultimate step in the enantioselective synthesis of the target Ritalin-methoxyamine derivative **317** is the dirhodium-catalyzed intermolecular C-H insertion reaction between *N*-Boc piperidine **306** and a transient metal carbene generated *in situ* by a metal-induced extrusion of nitrogen from α-diazo methyl ester **309**.[100] Thus, α-diazo methyl ester **309** and *N*-Boc piepridine **306** were allowed to react in the presence of catalytic amount of Rh_2(S-DOSP)$_2$ under an argon atmosphere at $-50\,^{o}$C (**Scheme 4.27**).



Scheme 4.27. Attempted dirhodium-catalyzed intermolecular C-H insertion reaction of α-diazo methyl ester **309** and *N*-Boc piperidine **306**. Reagents and conditions: a. Rh_2(S-DOSP)$_2$, hexane, Ar, 14 h, -50 ^{o}C.

TLC analysis of the reaction mixture indicated complete consumption of the starting α-diazo compound **300** after fourteen hours of reaction. However, a complex mixture of products was observed by TLC and in the ^{1}H NMR spectrum of the isolated product. Attempts at separating the crude mixture by conventional separation

techniques (chromatography and recrystallization) were unsuccessful. Despite the complexity of the crude product mixture, neither the starting α-diazo methyl ester **309** nor the estimated proton signals of the desired product were present in the ^{1}H NMR spectrum. As mentioned in the preceding section, the α-diazo methyl ester **309** is unstable and decomposes over time to give the methyl ester precursor **223**. However, this was not the case under the dirhodium-catalyzed intermolecular C-H insertion reaction conditions because the ^{1}H NMR spectrum of the crude mixture did not contain any characteristic proton signals that matched those of the methyl ester **223** protons.

In addition, HRMS analysis of the crude mixture further indicated the formation of the desired product **317** was unsuccessful. Attempts to optimize the reaction conditions including performing the reaction at different temperatures (-78 ^{o}C, room temperature and 50 ^{o}C), increasing or decreasing the catalytic load (from 1% mol to 5% or 0.5% mol) and using different solvents (cyclohexane) produced similar results. While no other dirhodium catalyst was employed, it would be interesting to examine other catalysts in the future.

4.3 Summary of Results

As part of a wider strategy towards developing novel dual action antioxidant nitroxides, the pharmacophore hybridization approach was successfully employed herein to synthesize the novel, merged L-Dopa nitroxide analogue **149**. The target chiral α-amino acid **149** was prepared via the Schöllkopf's bis-lactim ether asymmetric synthesis of α-amino acids. The key step in this approach involved diastereoselective alkylation of the Schöllkopf chiral auxiliary **275** with benzyl bromide intermediates (**280** and **283**) in high yields. As a potential dual acting therapeutic, it is anticipated that compound **149** could retain the beneficial anti-Parkinson's effects while at the same time provide remedy for the drug-induced cellular oxidative stress-related damages that usually accompanies PD drugs such as L-Dopa.

The Schöllkopf's bis-lactim ether approach in combination with the palladium-catalyzed Sonogashira coupling reaction was also successfully employed to

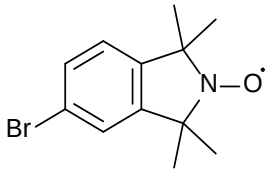
synthesize the rigid alkyne-linked chiral α-amino acid **151**. Due to its rigidity and chirality, compound **151** was designed as a potential site directed spin label for studying the structure and dynamics of macromolecules, specifically proteins, with minimal drawbacks associated with rotational mobility, isomerization, multiple conformers and achiral effects. As a potent radical scavenger, compound **151** could also be used to monitor ROS-induced cellular oxidative stress-related damages to peptides and proteins under study.

In addition to successfully synthesizing target compounds **149** and **151**, attempts were made to synthesize the Ritalin-nitroxide analogue **150** via a highly regio-, diastereoselective rhodium-catalyzed intermolecular C-H insertion of methyl phenyldiazoacetate **309** into *N*-Boc-piperidine **206** to give the D-threo-methylephenedate TMIO methoxyamine derivative **317**. Despite successful synthesis of the diazoacetate intermediate **309**, unfortunately, the intermolecular C-H insertion step produced a complex mixture of products. Evidence for the desired product **317** or the starting diazoacetate **209** could not be found in either the ^{1}H NMR or HRMS spectra of the isolated product. Attempts to optimize the reaction conditions were unsuccessful.

At present both targets α-amino acids (**149** and **151**) are being considered for appropriate biological evaluation of their therapeutic and/or spin labelling effects. The final results and discussion section of this thesis (**Chapter 5**) uses the pharmacophore hybridization approach to synthesize novel dual acting catechol and 1,3-benzodioxole-based isoindoline TMI and TMIO compounds (**154-161**). The target compounds (**154-161**) are the first examples of catechol-based nitroxides that could function as potential dual antioxidant therapeutics as well as synthetic templates to build functional materials.

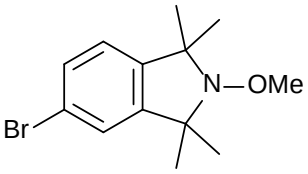
4.4 Experimental

4.4.1 Synthesis of 5-Bromo-1,1,3,3-tetramethylisoindolin-2-yloxyl 28



*m*CPBA (11 g, 37.8 mmol, 1.2 equiv, 77%) was added to a solution of 5-bromo-1,1,3,3-tetramethylisoindoline **66** (8 g, 31.5 mmol) in DCM (300 mL) at 0 ^{o}C. The reaction mixture was stirred for 30 min and then at RT for a further 1.5 h. The resulting solution was washed with dilute HCl (0.5 M, 50 mL), NaOH (2 M, 50 mL x 3) and brine (50 mL) solutions and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the residue purified by trituration from hexane to give compound **28** as a bright yellow solid (7.37 g, 87%). Mp. 138-140°C (Lit.,[50] 139-141°C).

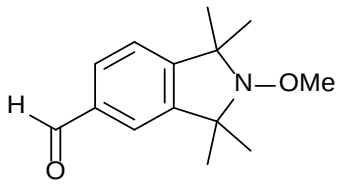
4.4.2 Synthesis of 5-Bromo-2-methoxy-1,1,3,3-tetramethylisoindoline 23



A solution of H_2O_2 (6.68 mL, 32 mmol, 2 equiv, 30%) was added dropwise (over 30 min) to a solution of 5-bromo-1,1,3,3-tetramethylisoindolin-2-yloxyl **28** (4.31 g, 16 mmol, 1 equiv.) and $FeSO_4.7H_2O$ (6.68 g, 24 mmol, 2 equiv.) in DMSO (40 mL). The resulting reaction mixture was stirred for 1 h and then diluted with H_2O (100 mL). The aqueous layer was extracted with Et_2O (70 mL x 4) and the combined Et_2O extracts were washed with NaOH (2 M, 50 mL), brine (50 mL), and then dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the crude residue purified by filtering through a short silica gel column (hexane) to give 5-bromo-2-methoxy-1,1,3,3-tetramethylisoindoline **23** as a colourless oil (4.232 g, 93%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.40 (br, s, 12 H, CH_3), 3.60 (s, 3 H, OCH_3), 6.90 (d, 1 H, *J* = 8.4 Hz, Ar-*H*), 7.20 (s, 1 H, Ar-*H*) 7.30 (d, 1 H, *J* = 8.4 Hz,

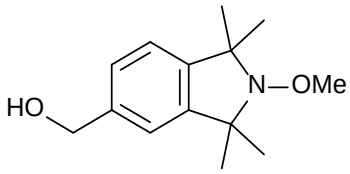
Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 25.0 (br, *C*H_3), 29.9 (br, *C*H_3), 65.5 (O*C*H_3), 66.9 (*C*-CH_3), 70.0 (*C*-CH_3), 120.7 (Ar-*C*), 123.3 (Ar-*C*), 124.8 (Ar-*C*), 130.2 (Ar-*C*), 144.2 (Ar-*C*), 147.5 (Ar-*C*).

4.4.3 Synthesis of 5-Formyl-2-methoxy-1,1,3,3-tetramethylisoindoline 22



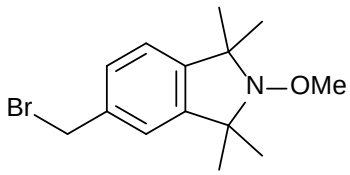
n-Butyllithium (3.57 mL, 5.72 mmol, 1.2 equiv, 1.6 M in hexanes) was added dropwise to a solution of 5-bromo-2-methoxy-1,1,3,3-tetramethylisoindoline **23** (1.354 g, 4.76 mmol, 1 equiv.) in dry THF (20 mL) at -78°C under Ar. DMF (1.1 mL, 14.3 mmol, 3 equiv.) was added after 15 min and the resulting mixture was allowed to warm to RT over 3 h. The reaction was quenched with H_2O (30 mL) and stirred for a further 15 min. The aqueous phase was extracted with Et_2O (60 mL x 4) and the combined Et_2O extracts were washed with HCl (2 M, 40 mL), NaOH (2 M, 40 mL), and brine (50 mL). The Et_2O layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (Hexane/DCM, 2:1) to afford 5-formyl-2-methoxy-1,1,3,3-tetramethylisoindoline **22** as white solid (1 g, 96%). Mp. 71-72 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (d, 6 H, C*H*$_3$), 1.46 (br, s, 6 H, C*H*$_3$), 3.78 (s, 3 H, OC*H*$_3$), 7.26 (d, 1 H, *J* = 7.8 Hz, Ar-*H*), 7.64 (d, 1 H, *J* = 1.2 Hz, Ar-*H*), 7.75 (dd, 1 H, *J* = 7.8 Hz, 1.2 Hz, Ar-*H*), 9.98 (s, 1 H, *H*C=O). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 24.4 (br, *C*H_3), 29.9 (br, *C*H_3), 65.6 (O*C*H_3), 67.0 (*C*-CH_3), 67.3 (*C*-CH_3), 122.2 (Ar-*C*), 122.6 (Ar-*C*), 130.1 (Ar-*C*), 136.1 (Ar-*C*), 146.5 (Ar-*C*), 152.5 (Ar-*C*), 192.0 (*C*=O). HRMS (ESI): *m*/*z* (%) = 234.1450 (20) $[M + H]^+$; calcd. for $C_{14}H_{19}NO_2$: 233.1416; found 233.1416. ATR-FTIR: υ_{max}= 2987 (m, Ar C-H), 1690 (s, C=O), 1048 (C-O) cm^{-1}.

4.4.4 Synthesis of 5-Hydroxymethyl-2-methoxy-1,1,3,3-tetramethylisoindoline 285



Sodium borohydride (175.1mg, 4.63 mmol, 1.5 equiv.) was added to a solution 5-formyl-2-methoxy-1,1,3,3-tetramethylisoindoline **23** (720 mg, 3.09 mmol, 1 equiv.) in methanol (10 mL) at 0 ^{o}C. The reaction mixture was stirred for 1.5 h and then concentrated *in vacuo*. The crude was residue picked up in Et_2O (60 mL x 4) and washed with brine (50 mL). The Et_2O layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure and the residue obtained was purified by purified by flash column chromatography (Hexane/EtOAc, 4:1) to afford 5-hydroxymethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **285** as white solid (701 g, 96%). Mp 63-64 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.43 (s, 12 H, CH_3), 3.78 (s, 3 H, OCH_3), 4.68 (s, 2 H, CH_2), 7.09 (d, 1 H, J = 7.8 Hz, Ar-*H*), 7.12 (s, 1 H, Ar-*H*), 7.23 (d, 1 H, J = 7.8 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 28.0 (w, br, C-CH_3), 30.0 (w, br, C-CH_3), 65.5 (OCH_3), 65.6 (CH_2), 67.0 (*C*-CH_3), 67.1 (*C*-CH_3), 120.3 (Ar-*C*), 121.7 (Ar-*C*), 126.2 (Ar-*C*), 140.0 (Ar-*C*), 144.9 (Ar-*C*), 145.7 (Ar-*C*). HRMS (ESI): *m*/*z* (%) = 236.1608 (15) $[M + H]^+$; calcd. for $C_{14}H_{21}NO_2$: 235.1572; found 235.1573. ATR-FTIR: υ_{max}= 3393 (br, OH), 2988 (m, Ar C-H), 1026 (C-O) cm^{-1}.

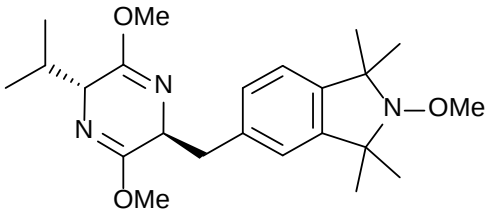
4.4.5 Synthesis of 5-Bromomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline 283



TEA (812 µL, 5.82 mmol, 2 equiv.) and then MsCl (450 µL, 5.82 mmol, 2 equiv.) were added dropwise to a solution of 5-hydroxymethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **285** (685 mg, 2.91 mmol, 1 equiv.) and LiBr (758 mg, 8.73 mmol, 3 equiv.) in freshly distilled THF (15 mL) at 0 ^{o}C under Ar. After stirring for

2 h, the resulting reaction mixture was then diluted with Et_2O (150 mL) and filtered. The filtrate was washed with brine (40 mL), dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. LiBr (505.6 mg, 5.82 mmol, 2 equiv.) was added to a solution of the crude residue in acetone (10 mL) and the resulting mixture was refluxed for 2 h. The reaction mixture was concentrated and taken up in Et_2O (60 mL x 4). The Et_2O layer was washed with brine (40 mL), dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. The residue obtained was purified on a short flash column (Hexane/Et_2O, 6:0.2) to give 5-bromomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **283** as a white solid (703 mg, 81%). Mp. 97-98 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.43 (s, 12 H, CH_3), 3.78 (s, 3 H, OCH_3), 4.51 (s, 2 H, CH_2), 7.06 (d, 1 H, J = 8 Hz, Ar-*H*), 7.11 (d, 1 H, J = 1.6 Hz, Ar-*H*), 7.26 (dd, 1 H, J = 8 Hz, 1.6 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 34.0 (*C*H_2), 65.5 (O*C*H_3), 67.0 (*C*-CH_3), 67.0 (*C*-CH_3), 121.9 (Ar-*C*), 122.2 (Ar-*C*), 128.2 (Ar-*C*), 136.8 (Ar-*C*), 145.7 (Ar-*C*), 145.9 (Ar-*C*). HRMS (ESI): *m*/*z* (%) = 298.0759/300.0751 (5) $[M + H]^+$; calcd. for $C_{14}H_{20}BrNO$: 297.0728; found 279.0726. ATR-FTIR: υ_{max}= 2971 (m, Ar C-H), 1051 (C-O), 653 (s, C-Br) cm^{-1}.

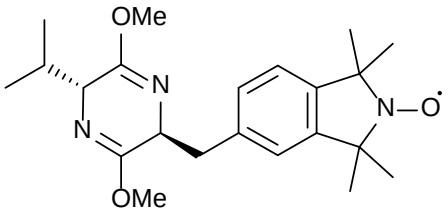
4.4.6 Synthesis of 5-(((2S,5R)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-2-methoxy-1,1,3,3-tetramethylisoindoline 287



A solution of (*R*)-2-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine **275** (300 µL, 1.67 mmol, 1 equiv.) in freshly distilled THF (10 mL) under Ar was cooled to -78 ^oC. *n*BuLi (1.1 mL, 1.76 mmol, 1.6 M, 1.05 equiv.) was added dropwise and the reaction mixture was stirred for 15 min. A solution 5-bromomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **283** (549 mg, 1.84 mmol, 1.1 equiv.) in THF (5 mL) was then added and stirring was continued for 3 h while allowing the reaction to return to RT. The reaction was quenched with H_2O and extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (40 mL), dried over anhydrous

Na_2SO_4 and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography (hexane/$CHCl_3$, 1:1) to give 5-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-2-methoxy-1,1,3,3-tetramethylisoindoline **287** as a white solid (535 mg, 80%). Mp. 62-63 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 0.59 (d, 3 H, *J* = 7.2 Hz, C*H*$_3$), 0.91 (d, 3 H, *J* = 7.2 Hz, C*H*$_3$), 1.38 (s, 12 H, C*H*$_3$), 2.11 (m, 1 H, C*H*), 3 (d, 1 H, C*H*), 3.07 (d, 2 H, *J* = 4.8 Hz, C*H*$_2$), 3.71 (s, 6 H, 2 x OC*H*$_3$), 3.77 (s, 3 H, OC*H*$_3$), 4.33 (q, 1 H, C*H*), 7.06 (d, 1 H, Ar-*H*), 7.11 (d, 1 H, Ar-*H*), 7.26 (dd, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 16.4 (*C*H$_3$), 19.0 (*C*H$_3$), 30.9 (*C*(CH$_3$)$_2$), 40.0 (*C*H$_2$), 52.1 (O*C*H$_3$), 52.4 (O*C*H$_3$), 56.8 (*C*H), 60.1 (*C*H), 65.4 (O*C*H$_3$), 66.89 (*C*-CH$_3$), 66.92 (*C*-CH$_3$), 120.9 (Ar-*C*), 123.4 (Ar-*C*), 128.8 (Ar-*C*), 136.0 (Ar-*C*), 143.3 (Ar-*C*), 144.5 (Ar-*C*), 162.4 (*C*=N), 164.2 (*C*=N). HRMS (ES): *m*/*z* (%) = 402.2775 (100) $[M + H]^+$; calcd. for $C_{23}H_{35}N_3O_3$: 401.2678; found 401.2679. ATR-FTIR: υ_{max}= 2971 (m, Ar C-H), 1695 (s, C=N), 1235 and 1224 (s, C-N), 1047 and 1016 (C-O) cm^{-1}.

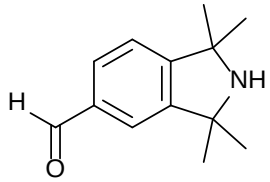
4.4.7 Synthesis of 5-(((2S,5R)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl 269 via oxidation of 281



$NaHCO_3$ (74 mg, 882 µmol, 1.5 equiv.) and *m*CPBA (177 mg, 882 µmol, 1.5 equiv, 77%) were added to a solution of 5-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-2-methoxy-1,1,3,3-tetramethylisoindoline **287** (236 mg, 588µmol, 1 equiv.) in DCM (20 mL) at 0 ^oC. The cooling bath was removed after 30 min and the reaction mixture was stirred at RT for further 2.5 h. The resulting solution was diluted (DCM, 100 mL), washed with NaOH (0.1 M, 30 mL), brine (40 mL) and then dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the crude residue obtained was purified by flash column chromatography (Hexane/EtOAc, 1:0.1) to give 5-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl

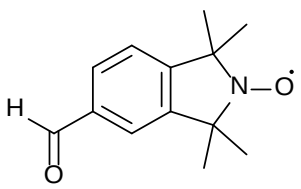
281 as a yellow solid (184 mg, 81%). Mp. 85-86 °C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.61 (br, s, 3 H, C*H*$_3$), 0.85 (br, s, 3 H, C*H*$_3$), 2.14 (br, s, 1 H, C*H*), 3.19 (br, s, 1 H, C*H*), 3.5 (br, t, 1 H, C*H*), 3.72 (br, s, 3 H, OC*H*$_3$), 3.73 (br, s, 3 H, OC*H*$_3$), 4.39 (br, s, 1 H, C*H*$_2$). HRMS (ES): *m*/*z* (%) = 409.2320 (70) [M + Na]$^+$; calcd. for $C_{22}H_{32}N_3O_3^{\bullet}$: 386.2444; found 386.2444. ATR-FTIR: υ_{max}= 2971 (m, Ar C-H), 1694 (s, C=N), 1235 and 1224 (s, C-N), 1047 and 1016 (C-O) cm^{-1}.

4.4.8 Synthesis of 5-Formyl-1,1,3,3-tetramethylisoindoline 73



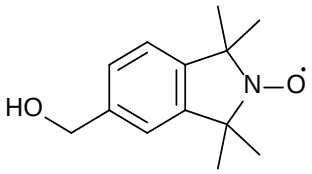
n-Butyllithium (1.6 M in hexanes, 5.45 cm^3, 8.72 mmol, 2.2 equiv.) was added dropwise to a solution of 5-bromo-1,1,3,3-tetramethylisoindoline **66** (1 g, 3.94 mmol) in dry THF (11 mL) at -78°C under argon. After 10 minutes the reaction was quenched by the addition of dry DMF (0.915 mL, 11.8 mmol, 3 equiv.). The resulting mixture was allowed to warm to room temperature and then diluted with H_2O (100 mL). The aqueous phase was acidified with HCl (2 M) and extracted with Et_2O (40 mL x 2) to remove non-basic impurities. The aqueous phase was then basified (pH >10) with NaOH (5 M) and extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (0.2% MeOH/$CHCl_3$) to afford compound **73** (800 mg, 100%) as an off-white crystalline solid. ^{1}H NMR ($CDCl_3$): δ = 1.50 (s, 6 H, 2 x C*H*$_3$), 1.51 (s, 6 H, 2 x C*H*$_3$), 7.29 (d, 1 H, *J* = 7.8 Hz, Ar-*H*), 7.68 (d, 1 H, *J* = 2.2 Hz, Ar-*H*), 7.80 (dd, 1 H, *J* = 7.8 Hz, 2.2 Hz, Ar-*H*), 10.03 (s, 1 H, O=C*H*).

4.4.9 Synthesis of 5-Formyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 27



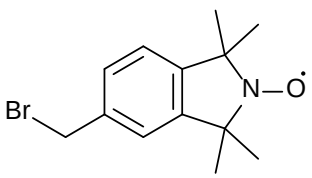
A solution of *m*CPBA (1.483 g, 5.09 mmol, 1.1 equiv, 77%) in DCM (10 mL) was added dropwise to a solution of 5-formyl-1,1,3,3-tetramethylisoindoline **73** (1 g, 4.62 mmol) in DCM (150 mL) at 0 ^{o}C. The reaction mixture was stirred for 2 h the resulting bright yellow solution was washed with dilute HCl (0.5 M, 40 mL), NaOH (2 M, 40 mL x 2) and brine solutions and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the residue purified by flash column chromatography (Hexane/EtOAc, 4:1) to give compound **27** as a bright yellow solid (928 mg, 92%). Mp. 138-140°C (Lit: 139-141°C).[101]

4.4.10 Synthesis of 5-hydroxymethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 280



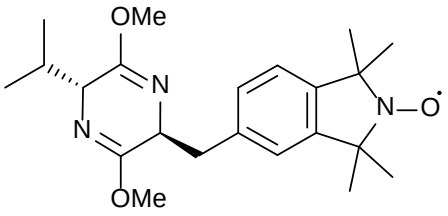
Sodium borohydride (60.31 mg, 1.59 mmol, 1.5 equiv.) was added to a solution 5-formyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **27** (232 mg, 1.06 mmol, 1 equiv.) in methanol (5 mL) at 0 ^{o}C and the reaction mixture was stirred for 1.5 h. The reaction mixture was concentrated *in vacuo* and the residue taken up in Et_2O (150 mL). The Et_2O layer was washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford 5-hydroxymethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **288** as a yellow solid which could be readily recrystallized from cyclohexane (200 mg, 86%). Mp. 110 (Lit.,[102] 108-110 ^{o}C). HPLC purity (>99%). FTIR (ATR): υ_{max} = 3425 (s, O-H), 1426 (m, N-O), 1027 (s, C-O) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 221.1373 (15) [M + H]; calcd. for $C_{13}H_{18}NO_2^{\bullet}$ 220.1338; found 220.1341.

4.4.11 Synthesis of 5-Bromomethyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 280



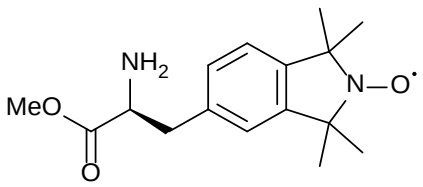
Compound **280** was obtained from **288** by following similar procedure as per **Section 4.4.5** above. Yellow solid, 75%. Mp. 110 (Lit.,[102] 108-110 ^{o}C). HPLC purity (>95%). HRMS (ESI): *m*/*z* (%) = 283.0488/285.0491 (30) $[M + H]^{+}$; calcd. for $C_{13}H_{17}BrNO^{\bullet}$: 282.0494; found 282.0497.

4.4.12 Synthesis of 5-(((2S,5R)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl 281 via benzyl bromide nitroxide 280



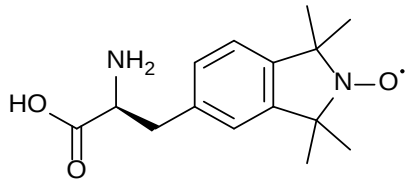
A solution of (*R*)-2-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine **275** (226 µL, 1.26 mmol, 1.1 equiv.) in freshly distilled THF (10 mL) under Ar was cooled to -78 ^{o}C. *n*BuLi (775 µL, 1.24 mmol, 1.6 M, 1.08 equiv.) was added dropwise and the reaction mixture was stirred for 15 min. A solution 5-bromomethyl-1,1,3,3-tetramethylisoindoline **280** (325 mg, 1.15 mmol, 1 equiv.) in THF (5 mL) was then added and stirring was continued for 3 h while allowing the reaction to return to RT. The reaction was quenched with H_2O and extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 1:0.1) to give 5-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl **281** yellow solid (346 mg, 78%). All data obtained were consistent with those from **Section 4.4.7**.

4.4.13 Synthesis of methyl (S)-2-Amino-3-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)propanoate 282



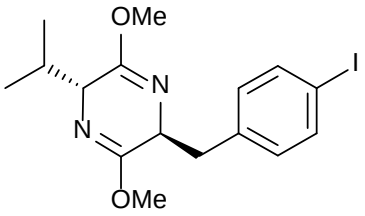
Dilute HCl (2 mL, 0.5 M) was added to a solution of 5-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)-1,1,3,3-tetramethylisoindolin-2-yloxyl **281** (170 mg, 440 µmol, 1 equiv.) in MeCN (5 mL) at 0 °C. The resulting reaction mixture was allowed to return to RT while stirring overnight and diluted with H_2O (20 mL). The aqueous layer was washed with DCM (15 mL), then cooled to 0 °C, and basified with saturated Na_2CO_3 solution to pH 11. The basic solution was extracted with DCM (30 mL x 4) and the combined DCM extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography (DCM/4%MeOH) to give (*S*)-2-amino-3-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)propanoate **282** as a yellow solid (96 mg, 76%). Mp. 88-90 °C. HPLC purity (>95%) HRMS (ES): *m/z* (%) = 292.1724 (70) $[M + H]^+$, 314.2094 (100) $[M + Na]^+$; calcd. for $C_{16}H_{23}N_2O_3^{\bullet}$: 291.1709; found 291.1709. ATR-FTIR: υ_{max}= 3382, 3319 (w, NH_2), 2974 (m, Ar C-H), 1730 (s, C=O), 1195 (s, CN), 1118 (s, C-O) cm^{-1}.

4.4.14 Synthesis of (S)-2-Amino-3-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)propanoic acid (L-Dopa-TMIO) 149



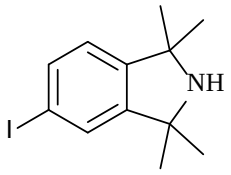
A solution of (*S*)-2-amino-3-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)propanoate **282** (78 mg, 268 µmol, 1 equiv.) in THF/H_2O (3 mL/1 mL) was cooled to 0 °C. LiOH (19.23 mg, 803 µmol, 3 equiv.) was added and the reaction mixture was stirred for 10 min and then at RT for 2 h. The resulting solution was concentrated under reduced pressure and the residue obtained was purified by trituration with THF to give (*S*)-2-amino-3-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)propanoic acid **149** as a yellow solid (96 mg, 76%). Mp. 121 °C (dec). HRMS (ES): *m/z* (%) = 300.1432 (20) $[M + Na]^+$; calcd. for $C_{15}H_{21}N_2O_3^{\bullet}$: 277.1552; found 277.1552. ATR-FTIR: υ_{max}= 3376 (s, O-H), 2985 (m, Ar C-H), 1630 (s, C=O), 1175 (s, C-O) cm^{-1}.

4.4.15 Synthesis of (2S,5R)-2-(4-Iodobenzyl)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine 296



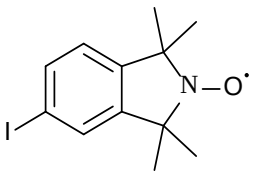
A solution of (*R*)-2-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine **275** (200 µL, 1.12 mmol, 1 equiv.) in freshly distilled THF (5 mL) under Ar was cooled to -78 ^{o}C. To that was added dropwise *n*BuLi (761 µL, 1.22 mmol, 1.6 M, 1.05 equiv.) and the reaction mixture was stirred for 15 min. Then a solution 4-iodobenzyl bromide **295** (398 mg, 1.34 μmol, 1.2 equiv.) in THF (3 mL) was added and stirring was continued for 1h at -78 ^{o}C before allowing it to return to room temperature (3 h). The reaction was quenched with H_2O (100 mL) and extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography (Hexane/EtOAc, 4:0.2) to give (2*S*,5*R*)-2-(4-iodobenzyl)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine **296** (384 mg, 86%) as a white solid. Mp. 96-96.5 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.61 (d, 3 H, *J* = 6.8 Hz, C*H*$_3$), 0.96 (d, 3 H, *J* = 6.8 Hz, C*H*$_3$), 2.16 (m, 1 H, C*H*), 3.02 (d, 1 H, *J* = 3 Hz, C*H*$_2$), 3.4 (d, 1 H, C*H*), 3.67 (s, 3 H, OC*H*$_3$), 3.71 (s, 3 H, OC*H*$_3$), 4.28 (d, 1 H, *J* = 4 Hz, C*H*), 6.85 (d, 2 H, *J* = 7.6 Hz, Ar-*H*), 7.53 (d, 2 H, *J* = 7.6 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 16.5 (*C*H$_3$), 19.0 (*C*H$_3$), 31.4 (*C*(CH$_3$)$_2$), 39.4 (*C*H$_2$), 52.2 (OCH$_3$), 52.4 (OCH$_3$), 56.3 (*C*H), 60.1 (*C*H), 91.8 (Ar-*C*), 132.0 (Ar-*C*), 136.9 (Ar-*C*), 137.1 (Ar-*C*), 162.2 (*C*=N), 164.0 (*C*=N). HRMS (ES): *m*/*z* (%) = 401.0653 (100) $[M + H]^+$; calcd. for $C_{16}H_{21}IN_2O_2$: 400.0648; found 400.0650. ATR-FTIR: υ_{max}= 2964 (m, Ar C-H), 1689 (s, C=N), 1224 (s, C-N), 1008 (C-O) cm^{-1}.

4.4.16 Synthesis of 5-Iodo-1,1,3,3-tetramethylisoindoline 68[81]



n-Butyllithium (1.6 M in hexane, 5.45 mL, 8.72 mmol, 2.2 equiv.) was added dropwise to a solution of 5-bromo-1,1,3,3-tetramethylisoindoline **66** (1 g, 3.94 mmol 1 equiv.) in dry THF (15 mL) at -78°C under argon. After 10 minutes the reaction was quenched by the addition of iodine (3g in 10 mL THF, 11.8 mmol, 3 equiv.) in dry THF (10 mL). The resulting mixture was allowed to warm to room temperature and then diluted with H_2O (40 mL). The aqueous phase was basified with NaOH (5 M) and extracted with DCM (60 mL x 4). The combined DCM extracts were washed with brine (40 mL) and the aqueous phase was then basified (pH >10) with NaOH (5 M) and extracted with DCM (60 mL x 4). The combined DCM extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue obtained was dissolved in MeOH/DCM (10:1 mL) and $NaHCO_3$ (100 mg) added. Aqueous hydrogen peroxide solution (1 mL, 30 wt. % in H_2O) was added dropwise and the resulting solution acidified with H_2SO_4 (30 mL, 2 M). Acid aqueous layer was washed with DCM (20 mL x 2) and the combined DCM layers were extracted with H_2SO_4 (3 x 15 mL, 2 M). The combined H_2SO_4 extracts were then washed with DCM (20 mL) and basified with NaOH (10 M). The basic layer was extracted with DCM (60 mL x 4) and the combined DCM layers were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 5-iodo-1,1,3,3-tetramethylisoindoline **68** as golden oil (950 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 6 H, C*H*$_3$), 1.44 (s, 6 H, C*H*$_3$), 6.9 (d, 1 H, Ar-*H*), 7.45 (d, 1 H, Ar-*H*), 7.56 (dd, 1 H, Ar-*H*). ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.7 (*C*H$_3$), 31.8 (*C*H$_3$), 62.6 (*C*CH$_3$), 62.7 (*C*CH$_3$), 92.2 (Ar-*C*), 123.5 (Ar-*C*), 130.8 (Ar-*C*), 136.0 (Ar-*C*), 138.6 (Ar-*C*), 151.4 (Ar-*C*).

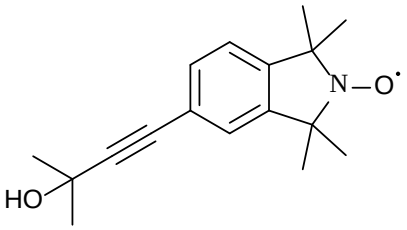
4.4.17 Synthesis of 5-Iodo-1,1,3,3-tetramethylisoindolin-2-yloxyl 61[80]



m-Chloroperoxybenzoic acid (1.3 g, 4.48 mmol, 1.5 equiv., 77%) was added to a solution of 5-iodo-1,1,3,3-tetramethylisoindoline **68** (900 mg, 3 mmol, 1 equiv.) in DCM (200 mL) at 0 ^{o}C. The reaction mixture was stirred for 30 min and then at RT for a further 2.5 h. The resulting solution was washed with saturated HCl (2 M, 40

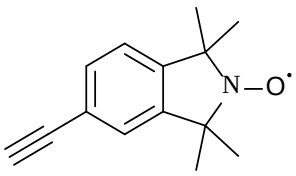
mL), NaOH (2 M, 40 mL x 2) and brine (50 mL) solutions and dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude residue purified by flash column chromatography (Hexane/DCM, 1:1) to give 5-iodo-1,1,3,3-tetramethylisoindolin-2-yloxyl **61** as a yellow solid (825 mg, 87%). Mp. 132-135 °C (Lit.,[81] 132-135 °C).

4.4.18 Synthesis of 5-(3-Hydroxy-3-methyl)butynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 297[80]



A reaction mixture of 5-iodo-1,1,3,3-tetramethylisoindolin-2-yloxyl **61** (800 mg, 2.53 mmol), DABCO (852 mg, 7.59 mmol, 3 equiv.), $Pd(OAc)_2$ (16 mg, 76 µmol, 3 mol%), 2-methyl-3-butyn-2-ol 9 (1.21 mL, 12.65 mmol, 5 equiv.) in MeCN (15 mL) was refluxed under Ar for 24 h. The mixture was concentrated *in vacuo* and the crude residue purified by silica gel flash column chromatography (Hexane/EtOAc, 1:1) to give 5-(3-hydroxy-3-methyl)butynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **297** as a brown–orange oil (482 mg, 70%). ATR-FTIR: υ_{max}= 3387 (OH), 2976 (ArCH) cm^{-1}.

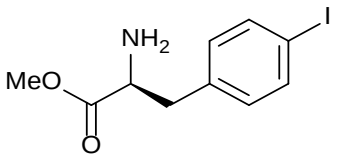
4.4.19 Synthesis of 5-Ethynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl 90



A suspension of KOH (924 mg, 14.3 16.52 mmol, 10 equiv.) in toluene (150 mL) was heated to reflux until the solid KOH was almost completely dissolved. 5-(3-Hydroxy-3-methyl)butynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **297** (450 mg, 1.65 mmol, 1 equiv.) in toluene (20 mL) was the added and the resulting solution was further refluxed for 30 min. The reaction mixture was then allowed to cool to RT

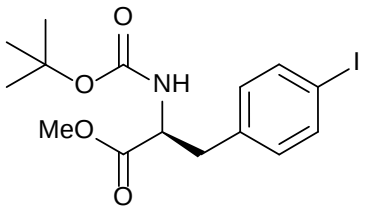
and washed with brine (15 mL x 2), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue obtained was purified by silica gel flash column chromatography (Hexane/EtOAc, 4:1) to give 5-ethynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **90** as a pale yellow crystalline solid (329 mg, 93%). Mp. 126–128 C (Lit.,[80] 126–128 °C).

4.4.20 Synthesis of (S)-Methyl 2-amino-3-(4-iodophenyl)propanoate hydrochloride 299



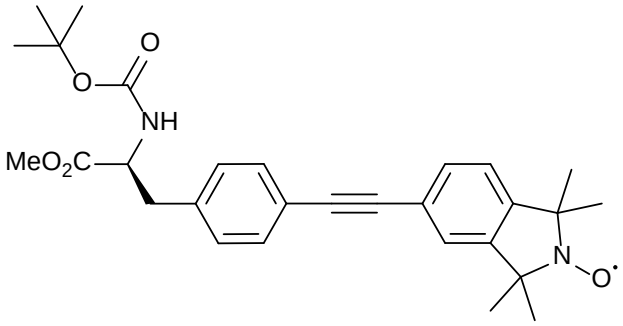
A solution of (2*S*,5*R*)-2-(4-iodobenzyl)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine **296** (220 mg, 550 µmol, 1 equiv.) in MeCN/H_2O (5 mL/0.5 mL) was cooled to 0 °C. A solution of TFA (128 µL, 1.65 mmol, 3 equiv.) in DCM (1 mL) was added dropwise and the resulting mixture was stirred at 0 °C for 30 min and then at RT for a further 2.5 h. The reaction mixture was then cooled in an ice bath and basified with saturated $NaHCO_3$ solution (ca. pH 10). The resulting mixture was extracted with DCM (60 mL x 4) and the combined DCM extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude residue was obtained as a mixture of (*S*)-4-iodophenylalanine **299** and D-valine **279** methyl esters as determined by ^{1}H NMR spectroscopy (140 mg). This crude mixture was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz): **Peaks for 299**; δ = 0.90 (d, 3 H, J = 4 Hz, CH_3), 0.97 (d, 3 H, J = 4 Hz, CH_3), 2.02 (m, 1 H, C*H*), 2.81 (dd, 1 H, J = 12 Hz, 4 Hz, CH_2), 3.03 (dd, 1 H, J = 12 Hz, 4 Hz, CH_2), 3.31 (d, 1 H, J = 4 Hz, C*H*), 3.72 (s, 3 H, OCH_3), 6.95 (d, 2 H, J = 8 Hz, Ar-*H*), 7.63 (d, 2 H, J = 8 Hz, Ar-*H*).[103]

4.4.21 Synthesis of methyl (S)-2-((tert-Butoxycarbonyl)amino)-3-(4-iodophenyl)propanoate 300



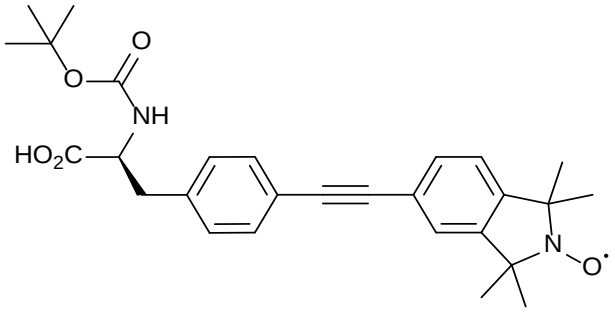
A mixture of (*S*)-4-iodophenylalanine **299** and D-valine **279** methyl esters (140 mg) in DCM (5 mL) was cooled in an ice bath. TEA (192 µL, 1.37 mmol, 2.5 equiv.) and then $(Boc)_2O$ (240 mg, 1.1 mmol, 2 equiv.) were added and the resulting mixture was stirred overnight while allowing it to return to RT. The reaction mixture was diluted with H_2O then extracted with DCM (60 mL x 4). The combined DCM extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated under vacuum. The crude residue obtained was purified by silica gel column chromatography (Hexane/EtOAc, 5:0.6) to give (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-iodophenyl)propanoate **300** as a white solid (140 mg, 66% over the two steps from **296**). Mp. 77.5-78 ^oC (Lit.,[104] 78.5-80 ^oC). HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (s, 9 H, $C(CH_3)_3$), 2.95 (dd, 1 H, *J* = 14.4 Hz, 6 Hz, CH_2), 3.06 (dd, 1 H, *J* = 14.4 Hz, 6.4 Hz, CH_2), 3.72 (s, 3 H, OCH_3), 4.56 (br, d, 1 H, C*H*), 4.96 (br, d, 1 H, N*H*), 6.87 (d, 2 H, *J* = 8 Hz, Ar-*H*), 7.61 (d, 2 H, *J* = 8 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 28.3 (CH_3), 37.92 (CH_2), 37.93 ($C(CH_3)_2$), 52.3 (OCH_3), 54.2 (*C*H), 92.5 (Ar-*C*), 131.3 (Ar-*C*), 135.7 (Ar-*C*), 137.6 (Ar-*C*), 155.0 (*C*=O), 172.1 (*C*=O). HRMS (ES): *m*/*z* (%) = 390.0489 (40) [M + H]$^+$; calcd. for $C_{15}H_{20}INO_2$: 389.0488; found 389.0488. ATR-FTIR: υ_{max}= 3343 (m, N-H), 2967 (m, Ar C-H), 1735 (s, C=O), 1684 (s, C=O), 1159 (C-O) cm^{-1}.

4.4.22 Synthesis of Methyl (S)-2-((tert-butoxycarbonyl)amino)-3-(4-((1,1,3,3-tetramethylisoindolin—2-yloxyl-5-yl)ethynyl)phenyl)propanoate 301



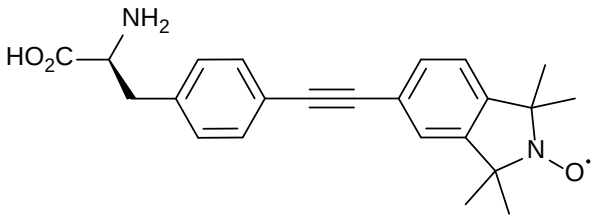
Degassed TEA (200 µL) was added to a degassed reaction mixture of (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-iodophenyl)propanoate **300** (100 mg, 247 µmol, 1 equiv.), 5-ethynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **90** (63.44 mg, 296 µmol, 1.2 equiv.), $Pd(PPh_3)_3$ (4.33 mg, 6.18 µmol, 0.025 equiv.), and CuI (1.17 mg, 6.18 µmol, 0.025 equiv.) in THF (10 mL) under Ar. The reaction mixture was stirred at RT for 1 d and then concentrated *in vacuo*. The crude residue obtained was purified by silica gel column chromatography (hexane/EtOAc, 5:1) to give methyl (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoate **301** as a yellow solid (81 mg, 66%). Mp. 183-184 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.43 (s, 9 H, C(C*H*$_3$)$_3$), 3.10 (d, 1 H, *J* = 3.6 Hz, C*H*$_2$), 3.15 (d, 1 H, *J* = 4 Hz, C*H*$_2$), 3.73 (s, 3 H, OC*H*$_3$), 4.6 (br, d, 1 H, *J* = 2.4 Hz, C*H*), 4.50 (br, d, 1 H, *J* = 7.2 Hz, N*H*), 7.15 (br, d, 2 H, *J* = 5.2 Hz, Ar-*H*), 7.44 (br, d, 2 H, *J* = 5.2 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 492.2455 (5) $[M + H]^+$, 493.2577 (15) $[M + 2H]^+$, 514.2381 (100) $[M + Na]^+$; calcd. for $C_{29}H_{35}N_2O_5^{\bullet}$: 491.2546; found 491.2450. ATR-FTIR: υ_{max}= 3336 (w, N-H), 2975 (m, Ar C-H), 1744 (s, C=O), 1711 (s, C=O), 1161 (C-O) cm^{-1}.

4.4.23 Synthesis of (S)-2-((tert-Butoxycarbonyl)amino)-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoic acid 302



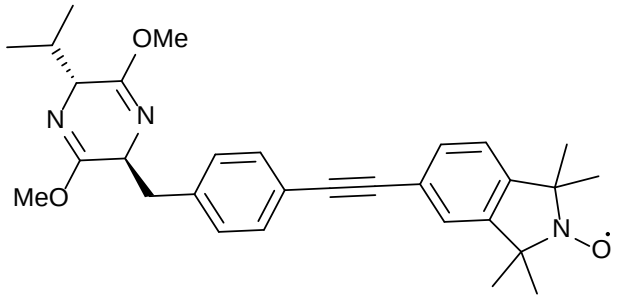
LiOH (6.44 mg, 269 µmol, 3 equiv.) was added to a solution of (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl) propanoate **301** (44 mg, 90 µmol, 1 equiv.) in H_2O/THF (1 mL/3 mL). The reaction mixture was stirred for 2 h and then diluted with H_2O (15 mL). The resulting solution was washed with Et_2O (15 mL) and the Et_2O layer discarded. The aqueous layer was cooled in ice bath, neutralized with saturated NH_4Cl, acidified with HCl (0.5 M, pH 3) and then extracted with EtOAc (40 mL x 4). The combined EtOAc extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo* give (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoic acid **302** as yellow solid (40 mg, 93%). Mp. 70-70.5 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.43 (s, 9 H, C(C*H*$_3$)$_3$), 3.14 (br, s, 1 H, C*H*$_2$), 3.25 (br, s, 1 H, C*H*$_2$), 4.59 (br, s, 1 H, C*H*), 4.98 (br, s, 1 H, N*H*), 7.22 (br, s, 2 H, Ar-*H*), 7.45 (br, s, 2 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 462.2445 (20) $[M + H]^+$; calcd. for $C_{28}H_{33}N_2O_4^{\bullet}$: 461.2440; found 461.2440. ATR-FTIR: υ_{max}= 3500-2500 (m, br, O-H), 2975 (m, Ar C-H), 1710 (s, br, C=O), 1161 (C-O) cm^{-1}.

4.4.24 Synthesis of (S)-2-Amino-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoic acid 151



HCl (0.2 mL, 4 M in dioxane) was added to a solution of (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoic acid **302** (35 mg, 73 µmol, 1 equiv.) in dioxane/H_2O (2 mL/0.1 mL). The reaction mixture was stirred overnight and concentrated *in vacuo*. The crude residue was washed with Et_2O (5 mL), dissolved in MeOH (2 mL) and precipitated by dropwise addition of THF. The precipitate was collected to give (*S*)-2-amino-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoic acid **151** as a yellow solid (29 mg, 96%). Mp. 162-163 ^oC (dec.). HRMS (ES): *m*/*z* (%) = 379.1989 (100) $[M + 2H]^+$; calcd. for $C_{23}H_{25}N_2O_3^{\bullet}$: 475.2597; found 475.2597. ATR-FTIR: υ_{max}= 3384 (s, br, OH), 2974 (m, Ar C-H), 1614 (s, C=O), 1119 (C-O) cm^{-1}.

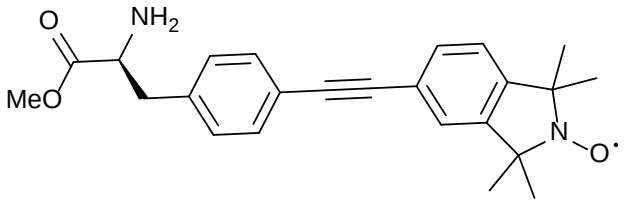
4.4.25 Synthesis of 5-[4-(((2S,5R)-5-Isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)phenyl)ethynyl] -1,1,3,3-tetramethylisoindolin-2-yloxyl 304



Degassed TEA (300 µL) was added to a degassed reaction mixture of (2*S*,5*R*)-2-(4-iodobenzyl)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazine **296** (150 mg, 374 µmol, 1 equiv.), 5-ethynyl-1,1,3,3-tetramethylisoindolin-2-yloxyl **90** (120 mg, 560 µmol, 1.5 equiv.), $PdCl_2(PPh_3)_2$ (13 mg, 19 µmol, 0.05 equiv.) and CuI (3.5 mg, 19 µmol, 0.05 equiv.) in THF (10 mL) under Ar. The reaction mixture was stirred at RT overnight and then concentrated *in vacuo*. The crude residue obtained was purified by silica gel column chromatography (Hexane/EtOAc, 4:0.4) to give 5-[4-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)phenyl)ethynyl]-1,1,3,3-tetramethylisoindolin-2-yloxyl **304** as a yellow solid (133 mg, 73%). Mp. 85-86 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 0.62 (d, 3 H, *J* = 6.8 Hz, C*H*$_3$), 0.96 (d, 3 H, *J* = 6.8 Hz, C*H*$_3$), 2.17 (br, d, 1 H, *J* = 3.2 Hz, C*H*), 3.14 (br, d, 2 H, *J* = 4.8 Hz, C*H*$_2$), 3.38 (br, s, 1 H, C*H*), 3.69 (s, 3 H, OC*H*$_3$), 3.73 (s, 3 H, OC*H*$_3$),

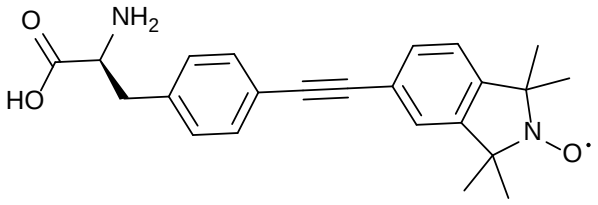
4.34 (br, d, 1 H, *J* = 4 Hz, C*H*), 7.11 (br, d, 2 H, *J* = 6.4 Hz, Ar-*H*), 7.37 (br, d, 2 H, *J* = 6.4 Hz, Ar-*H*). HRMS (ES): *m*/*z* (%) = 487.2791 (100) $[M + H]^+$, 509.2616 (60) $[M + Na]^+$; calcd. for $C_{30}H_{36}N_3O_3^{\bullet}$: 486.2757; found 486.2758. ATR-FTIR: υ_{max}= 2972 (m, Ar C-H), 1693 (s, C=N), 1237 (C-N), 1013 (C-O) cm^{-1}.

4.4.26 Synthesis of methyl (S)-2-Amino-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoate 305



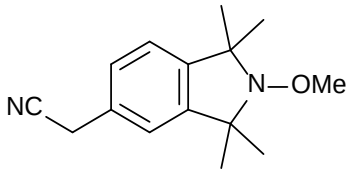
Aqueous HCl (0.1 mL, 1 M) was added to a solution of 5-[4-(((2*S*,5*R*)-5-isopropyl-3,6-dimethoxy-2,5-dihydropyrazin-2-yl)methyl)phenyl)ethynyl]-1,1,3,3-tetramethylisoindolin-2-yloxyl **304** (75 mg, 154 µmol, 1 equiv.) in MeCN (3 mL) and the reaction mixture was stirred vigorously for 2 h. The reaction mixture was diluted with H_2O and washed with DCM. The DCM wash layer was discarded and the aqueous layer was cooled in ice bath, basified with saturated $NaHCO_3$ (pH 10) and extracted with DCM (40 mL x 4). The combined DCM extracts were wash with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude residue obtained was purified by silica gel column chromatography (DCM/MeOH, 96:4) to give methyl (*S*)-2-amino-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl) propanoate **305** as a yellow solid (55 mg, 92%). Mp. 120-121 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 2.29 (br, dd, 1 H, *J* = 13.6 Hz, 7.6 Hz, CH_2), 3.13 (br, dd, 1 H, *J* = 13.6 Hz, 5.2 Hz, CH_2), 3.73 (s, 3 H, OCH_3), 3.76 (br, s, 1 H, C*H*), 7.22 (br, d, 2 H, Ar-*H*), 7.46 (br, d, 2 H, Ar-*H*). HRMS (ES): *m*/*z* (%) = 392.2055 (100) $[M + H]^+$, 414.1875 (40) $[M + Na]^+$; calcd. for $C_{24}H_{27}N_2O_3^{\bullet}$: 391.2022; found 391.2022. ATR-FTIR: υ_{max}= 3369 and 3309 (m, NH_2), 2972 (m, Ar C-H), 1741 (s, C=O), 1167 (C-O) cm^{-1}.

4.4.27 Synthesis of (S)-2-Amino-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoic acid 151



LiOH (3.7 mg, 153 µmol, 2 equiv.) was added to a solution of methyl (*S*)-2-amino-3-(4-((1,1,3,3-tetramethylisoindolin-2-yloxyl-5-yl)ethynyl)phenyl)propanoate **305** (30 mg, 77 µmol, 1 equiv.) in H_2O/THF (0.1 mL/3 mL). The reaction mixture was stirred for 2 h and then acidified to pH 3 with HCl (2 M) to give a compound **151** as a yellow precipitate that was filtered off. The remaining **151** was recovered by removing the solvent and triturating with cold MeOH/THF to removed NaCl. Compound **151** was obtained as a yellow solid (25 mg, 86%). Mp. 162-163 ^{o}C (dec.). HRMS (ES): *m/z* (%) = 379.1989 (100) $[M + 2H]^{+}$; calcd. for $C_{23}H_{25}N_2O_3^{\bullet}$: 475.2597; found 475.2597. ATR-FTIR: υ_{max}= 3384 (s, br, OH), 2974 (m, Ar C-H), 1614 (s, C=O), 1119 (C-O) cm^{-1}.

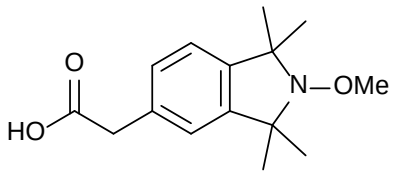
4.4.28 Synthesis of 5-Cyanomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline 311



TMSCN (201.5 µL, 1.61 mmol, 2 equiv.) was added drop wise to a solution of 5-bromomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **283** (240 mg, 805 µmol, 1 equiv.) and TBAF (1.61 mL, 1.61 mmol, 2 equiv., 1 M solution in THF) in MeCN (8 mL) under Ar, and the reaction mixture refluxed for 3 h and allowed to cool to RT. The mixture was diluted with Et_2O (150 mL) and washed with HCl (0.1 M, 20 mL), NaOH (0.1 M, 20 mL) and brine (40 mL), and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by a flash column chromatography (Eluent: Hexane/EtOAc, 5:1) to give 5-cyanomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **311** as a white solid (159 mg, 81%). Mp. 57-

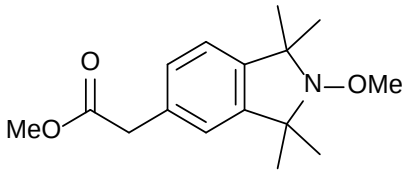
58 °C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.43 (br, s, 12 H, C*H*$_3$), 3.74 (s, 2 H, C*H*$_2$), 3.78 (s, 3 H, OC*H*$_3$), 7.05 (s, 1 H, Ar-*H*), 7.10 (d, 1 H, *J* = 8 Hz, Ar-*H*), 7.18 (d, 1 H, *J* = 8 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 23.6 (*C*H$_2$), 25.0 (br, C-*C*H$_3$), 29.0 (w, br, C-*C*H$_3$), 65.5 (O*C*H$_3$), 66.9 (*C*-CH$_3$), 67.1 (*C*-CH$_3$), 118.0 (*C*≡N), 121.1 (Ar-*C*), 122.3 (Ar-*C*), 127.0 (Ar-*C*), 128.8 (Ar-*C*), 145.3 (Ar-*C*), 146.4 (Ar-*C*). HRMS (ES): *m*/*z* (%) = 245.1582 (30) $[M + H]^+$; calcd. for $C_{15}H_{20}N_2O$: 244.1576; found 244.1576. ATR-FTIR: υ_{max}= 2972 (m, Ar C-H), 2250 (m, C≡N), 1051(s, C-O) cm^{-1}.

4.4.29 Synthesis of 5-Carboxymethyl-2-methoxy-1,1,3,3-tetramethylisoindoline 225 via hydrolysis of 311



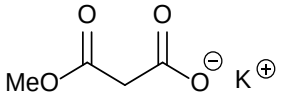
Conc. H_2SO_4 (3 mL, 98%) was added to a suspension of 5-cyanomethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **311** (250 mg, 1.03 mmol, 1 equiv.) in H_2O (3 mL). and the reaction mixture was refluxed overnight. The reaction mixture was cooled in an ice bath and diluted with water. The aqueous layer was extracted with Et_2O (60 mL x 4) and the combined Et_2O extracts were washed with brine (40 mL), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The crude residue was purified by a short silica gel column chromatography (EtOAc) to give 5-carboxymethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **225** as a white solid (226 mg, 84%). Mp. 115-116 °C. HPLC purity (>94%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (br, s, 12 H, C*H*$_3$), 3.63 (s, 2 H, C*H*$_2$), 3.77 (s, 3 H, OC*H*$_3$), 7.00 (s, 1 H, Ar-*H*), 7.04 (d, 1 H, *J* = 7.6 Hz, Ar-*H*), 7.15 (d, 1 H, *J* = 7.6 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 25.0 (br, C-*C*H$_3$), 29.0 (w, br, C-*C*H$_3$), 40.9 (*C*H$_2$), 65.5 (O*C*H$_3$), 66.9 (*C*-CH$_3$), 67.0 (*C*-CH$_3$), 121.7 (Ar-*C*), 122.5 (Ar-*C*), 128.3 (Ar-*C*), 132.2 (Ar-*C*), 144.4 (Ar-*C*), 145.7 (Ar-*C*), 176.9 (*C*=O). HRMS (ES): *m*/*z* (%) = 264.1519 (15) $[M + H]^+$; calcd. for $C_{15}H_{21}NO_3$: 263.1521; found 263.1521. ATR-FTIR: υ_{max}= 3382 (s, br, OH), 1973 (m, ArC-H), 1737 (s, C=O), 1158 (s, C-O) cm^{-1}.

4.4.30 Synthesis of 2-Methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindoline 223 from 225



A solution of 5-carboxymethyl-2-methoxy-1,1,3,3-tetramethylisoindoline **225** (100 mg, 380 µmol, 1 equiv.) and 2 drops of H_2SO_4 (98%) in MeOH (5 mL) was refluxed overnight. The reaction mixture was cooled to RT and diluted with H_2O (30 mL). The aqueous layer was extracted Et_2O (60 mL x 4) and the organic extract was washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude residue obtained was purified by silica gel column chromatography (Hexane/EtOAc, 5:0.2) to give 2-methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindolin **223** as a white solid (92 mg, 87%). Mp. 79-80 ^oC. HPLC purity (>99%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (br, s, 12 H, CH_3), 3.61 (s, 2 H, CH_2), 3.69 (s, 3 H, OCH_3), 3.77 (s, 3 H, OCH_3), 7.00 (s, 1 H, Ar-*H*), 7.05 (d, 1 H, *J* = 8 Hz, Ar-*H*), 7.14 (d, 1 H, *J* = 8 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 25.3 (w, br, *C*H_3), 29.7 (w, br, *C*H_3), 41.2 (*C*H_2), 52.1 (O*C*H_3), 65.5 (O*C*H_3), 66.9 (*C*-CH_3), 67.1 (*C*-CH_3), 121.7 (Ar-*C*), 122.4 (Ar-*C*), 128.2 (Ar-*C*), 132.9 (Ar-*C*), 144.1 (Ar-*C*), 145.6 (Ar-*C*), 172.2 (*C*=O). HRMS (ES): *m*/*z* (%) = 278.1680 (15) $[M + H]^+$; calcd. for $C_{16}H_{23}NO_3$: 277.1678; found 277.1678. ATR-FTIR: υ_{max}= 2975 (m, Ar C-H), 1736 (s, C=O), 1206 and 1143 (s, C-O) cm^{-1}.

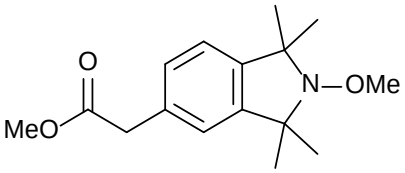
4.4.31 Synthesis of Methyl Potassium Malonate 316



A solution of methyl malonate **315** (5 mL, 43.75 mmol, 1 equiv.) in MeOH (30 mL) was cooled to 0 ^oC. KOH (2.455 g, 43.75 mmol, 1 equiv.) was added and the reaction mixture was allowed to return to RT while stirring overnight. The resulting solution was concentrated under reduced pressure and the residue obtained was washed with Et_2O and dried in vacuum oven overnight (40 ^oC) to give methyl potassium malonate

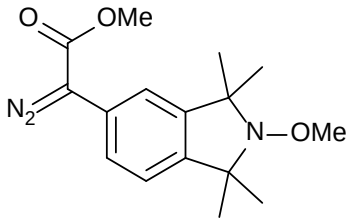
316 as a white solid (6.63 g, 97%). ^{1}H NMR (CD_3OD, 400 MHz): 3.33 (s, 3 H, OC*H*$_3$), 4.65 (s, 2 H, C*H*$_2$). ^{13}C NMR (D_2O, 100 MHz): δ = 44.4 (*C*H$_2$), 52.5 (O*C*H$_3$), 172.1 (*C*=O), 174.2 (*C*=O).

4.4.32 Synthesis of 2-Methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindoline 223 from 23 and 316



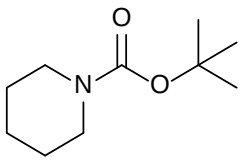
A Schlenk tube containing 5-bromo-2-methoxy-1,1,3,3-tetramethylisoindoline **23** (316 mg, 1.11 mmol, 1 equiv.), potassium methyl malonate **316** (434.13 mg, 2.78 mmol, 2.5 equiv.), allylpalladium (II) chloride dimer (8.14 mg, 22 µmol, 0.02 equiv.), BINAP (41.54 mg, 67 µmol, 0.06 equiv.), and DMAP (13.6 mg, 11 µol, 0.01 equiv.) in mesitylene (10 mL) was degassed and then heated for 1 d at 140 ^{o}C. The resulting mixture was concentrated under reduced pressure. The crude residue obtained was purified by silica gel column chromatography (Hexane/EtOAc, 5:0.2) to give 2-methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindoline **223** as a white solid (164 mg, 54%). Mp. 79-80 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (br, s, 12 H, C*H*$_3$), 3.61 (s, 2 H, C*H*$_2$), 3.69 (s, 3 H, OC*H*$_3$), 3.77 (s, 3 H, OC*H*$_3$), 7.00 (s, 1 H, Ar-*H*), 7.05 (d, 1 H, *J* = 8 Hz, Ar-*H*), 7.14 (d, 1 H, *J* = 8 Hz, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 25.3 (w, br, *C*H$_3$), 29.7 (w, br, *C*H$_3$), 41.2 (*C*H$_2$), 52.1 (O*C*H$_3$), 65.5 (O*C*H$_3$), 66.9 (*C*-CH$_3$), 67.1 (*C*-CH$_3$), 121.7 (Ar-*C*), 122.4 (Ar-*C*), 128.2 (Ar-*C*), 132.9 (Ar-*C*), 144.1 (Ar-*C*), 145.6 (Ar-*C*), 172.2 (*C*=O). HRMS (ES): *m*/*z* (%) = 278.1680 (15) $[M + H]^+$; calcd. for $C_{16}H_{23}NO_3$: 277.1678; found 277.1678. ATR-FTIR: υ_{max}= 2975 (m, Ar C-H), 1736 (s, C=O), 1206 and 1143 (s, C-O) cm^{-1}.

4.4.33 Synthesis of Methyl 2-diazo-2-(2-methoxy-1,1,3,3-tetramethylisoindolin-5-yl)acetate 309



DBU (116 µL, 772 µmol, 1.2 equiv.) was added dropwise to a solution of 2-methoxy-5-methoxycarbonylmethyl-1,1,3,3-tetramethylisoindoline **223** (179 mg, 644 µmol, 1 equiv.) and 4-acetamidobenzenesulfonyl azide (178 mg, 740 µmol, 1.15 equiv.) in MeCN (3 mL) under Ar. The resulting reaction mixture was stirred for 1 day and then concentrated under reduced pressure. The residue obtained was purified by silica gel column chromatography (Hexane/EtOAc, 8:0.2) to afford 2-diazo-2-(2-methoxy-1,1,3,3-tetramethylisoindolin-5-yl)acetate **309** as a bright yellow solid (168 mg, 86%). Mp. 91-92 ^{o}C. HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.41(br, s, 12 H, C*H*$_3$), 3.76 (s, 3 H, OC*H*$_3$), 3.85 (s, 3 H, OC*H*$_3$), 7.10 (d, 1 H, *J* = 8 Hz, Ar-*H*), 7.25 (d, 1 H, *J* = 8 Hz, Ar-*H*), 7.27 (s, 1 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 25.0 (w, br, C-*C*H$_3$), 30.0 (w, br, C-*C*H$_3$), 52.0 (O*C*H$_3$), 65.5 (O*C*H$_3$), 66.9 (*C*-CH$_3$), 67.2 (*C*-CH$_3$), 77.2 (N$_2$+=*C*), 117.6 (Ar-*C*), 122.1 (Ar-*C*), 123.2 (Ar-*C*), 124.1 (Ar-*C*), 143.4 (Ar-*C*), 146.2 (Ar-*C*), 165.8 (*C*=O). HRMS (ES): *m*/*z* (%) = 304.1591 (10) [M + H]$^{+}$; calcd. for $C_{16}H_{21}N_3O_3$: 303.1583; found 303.1583. ATR-FTIR: υ_{max}= 2970 (m, Ar C-H), 2107 (s, C=N$_2$), 1691 (s, C=O), 1047 (s, C-O) cm^{-1}.

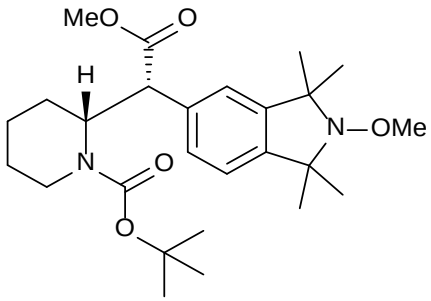
4.4.34 Synthesis of N-Boc Piperidine 306



A solution of di-*tert*-butyl dicarbonate (3 g, 13.75 mmol, 1 equiv.) in THF (15 mL) under an Ar atmosphere was cooled to 0 ^{o}C. Piperidine **310** (1.63 mL, 16.49 mmol, 1.2 equiv.) was added dropwise and the resulting solution stirred at RT for 2 h. 10% $NaHCO_3$ solution was added and the aqueous layer was extracted with Et_2O (60 mL

x 4). The Et_2O extract was washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue obtained was purified by Kugelrohr distillation to give compound **306** as colourless oil (2.42 mg, 95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.43(s, 9 H, CH_3), 1.48 (m, 4 H, CH_2), 1.53 (t, 2 H, CH_2), 3.33 (t, 4 H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 24.5 (*C*H_3), 25.7 (*C*H_2), 28.5 (*C*H_2), 44.6 (O*C*Me_3), 79.1 (N-*C*H_2), 154.9 (*C*=O).

4.4.35 Attempted Synthesis of tert-Butyl (R)-2-((R)-2-methoxy-1-(2-methoxy-1,1,3,3-tetramethylisoindolin-5-yl)-2-oxoethyl)piperidine-1-carboxylate 317



A solution of Rh_2(S-DOSP)$_4$ (3.125 mg, 1.65 μmol, 0.01 equiv.) and *N*-Boc piperidine **306** (63.4 μL, 330 μmol, 2 equiv.) in dry hexane (1 mL) was added dropwise (over 30 min) to a solution of 2-diazo-2-(2-methoxy-1,1,3,3-tetramethylisoindolin-5-yl)acetate **309** (50 mg, 165 μmol, 1 equiv.) in hexane (2 mL) at -50 ^{o}C under Ar. The resulting reaction mixture was stirred for 14 h at -50 ^{o}C and concentrated *in vacuo*. Attempts to purify the crude residue with silica gel column chromatography (Hexane/EtOAc, 4:1), trituration or recrystallization from methanol were unsuccessful.

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Chapter 5: Synthesis of Novel Catechol-Based Nitroxides as Potential Multifunctional Antioxidants

5.1 Background

The catechol moiety is an integral structural and functional subunit of a number of biologically relevant endogenous compounds. Catecholamine neurotransmitters such as adrenaline **319**, noradrenaline, and dopamine **152** (**Figure 5.1**), are involved in various cell signalling pathways.

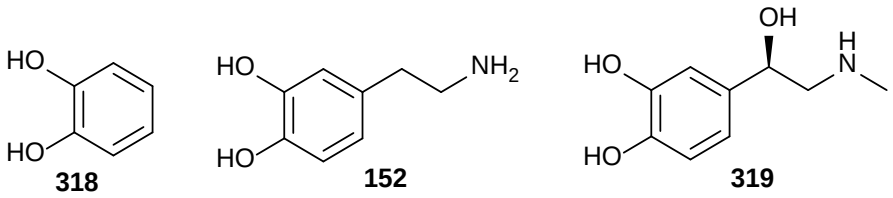
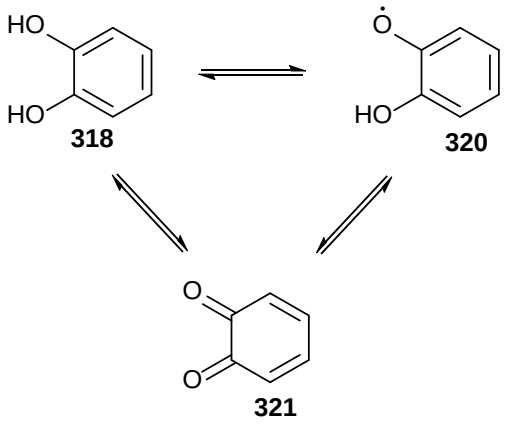


Figure 5.1. Chemical structure of catechol **318**, dopamine **152** and adrenaline **319**.

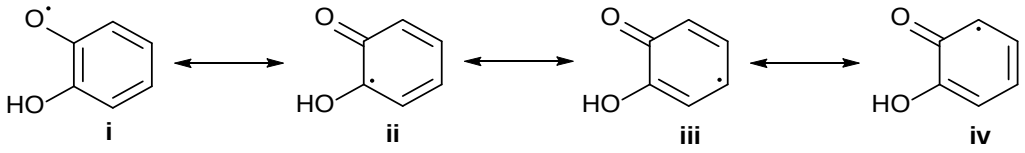
Most natural antioxidant compounds, particularly those derived from fruits and vegetables, are rich in catecholic functionality. Flavonoids for instance are a class of phenolic compounds isolated from edible plants.[1] They possess a variety of biological activities including antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory actions.[1-4] Polyphenolic antioxidants like catechols exert their antioxidant effect mainly via a radical scavenging action that involves redox chemistry with the phenol moiety. As depicted in **Scheme 5.1** below, the antioxidant activity of catecholinic compounds is attributed to the reversible redox triad between the catechol moiety **318** and its oxidation products, namely the semiquinone **320** and the quinone **321** derivatives.[5]

Although catechols do spontaneously oxidize (autoxidation) under standard atmospheric conditions,[6] the reaction is usually slow and most commonly, enzymes (such as horseradish peroxidase-hydrogen peroxide, HRP/H_2O_2)[7] and strong

oxidizing agents (sodium periodate, $NaIO_4$)[8] are employed for more rapid oxidation of catechols to their quinone counterparts. The resonance stabilized nature of the semiquinone radical **320** further accounts for the increased antioxidant action of catechol compounds (**Scheme 5.2**).[9]



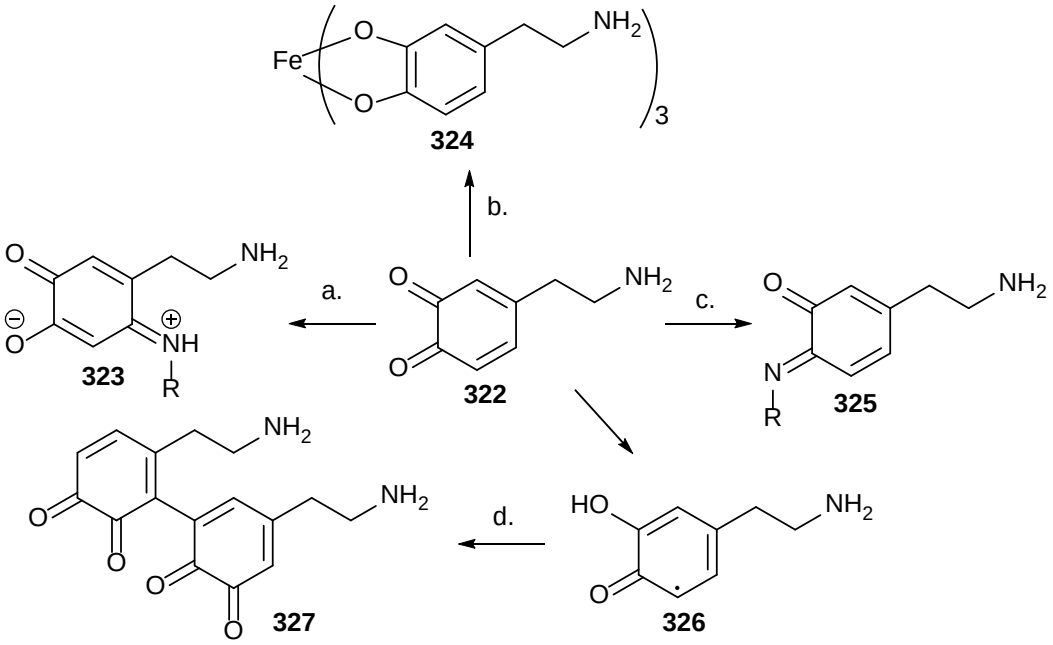
Scheme 5.1. Redox triad of catechols: catechol **318**, semiquinone **320** and quinone **321**.



Scheme 5.2. Resonance hybrids of the semiquinone radical **320**.

Owing to their electrophilic and redox characteristics, the highly reactive quinone species are extensively used as substrates to further modify and functionalize materials (**Scheme 5.3**). Such catechol-based functional materials are built exclusively via irreversible covalent bonds such as a Michael-type addition reaction to form thioethers and alkyl/aryl substituted amines, or a radical mediated homocoupling to form polymeric catechols.[10] Catechols, in the form of polydopamine-based quinones like **327** for example, are identified as the integral component of the protein secreted and used by mussels for attachment to a variety of surfaces.[11] Such strong adhesive properties of polydopamine have been utilized to build novel bioinspired and versatile adherent materials that can bind to both organic and inorganic surfaces.[12-17] For instance, Jiang and co-workers reported the synthesis

of modified zwitterionic poly(carboxybetaine)-catechol polymer surfaces (Au@pCB_2-catechol$_2$) which were used to directly immobilize anti-activated leukocyte cell adhesion molecules. [18]

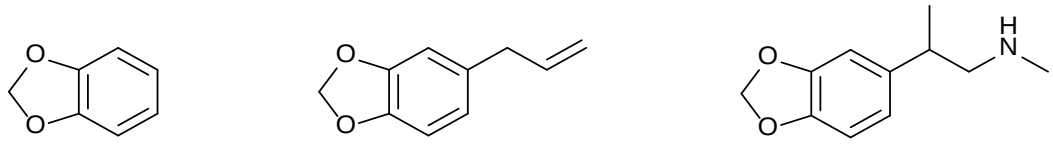


Scheme 5.3. Dopaquinone residue **322** as a substrate for building functional materials: a. Michael type addition reaction; b. Metal chelation; c. Schiff base reaction with amines (imine formation); d. Oxidative coupling reaction. Adapted and modified from Deming *et al.*[10,19,20]

Catechols and their *o*-quinone derivatives also serve as ideal substrates that form stable functional complexes **324** with a variety of di and trivalent metal ions.[20] Such complexes are commonly employed to build magnetic, sensing and electronic devices.

Like their catechol counterparts, the methylenedioxyphenyl group (or 1,3-benzodioxole) **328** is widely distributed in nature and it is also found in many pharmaceutical products (**Figure 5.2**).[21-23] Safrole **329** is a benzodioxole containing natural product used as a starting material to synthesize insecticides, fragrances and some drugs.[24-31] The illicit drug 3,4-methylenedioxy-*N*-methylamphetamine **153**, widely known as Ecstasy or MDMA, is a benzodioxole derivative synthesized from

Safrole **329**.[32] MDMA has strong psychotherapeutic effects which make it a common recreational drug that is widely abused by young people.[33-37]



328 **329** **153**

Figure 5.2. Chemical structures of common 1,3-benzodioxole compounds: parent 1,3-benzodioxole **328**, safrole **329** and MDMA **153**.

Benzodioxole is primarily generated by a base-catalyzed methylation of catechol with a dihalomethane. Both the stable electron rich aromatic ring and the methylenedioxy protecting group of benzodioxole containing compounds account for their lower polarity compared to their catechol counterparts. Such low polarity makes benzodioxole-based therapeutics much effective at crossing the blood-brain barrier. Like an acetal group, benzodioxole hydrolyzes to catechol under acidic conditions. Compounds with benzodioxole functionality are liable to be readily oxidized by enzymes and other oxidizing agents. In biological systems, the cytochrome P450 enzymes frequently catalyze the oxidative cleavage of the methylenedioxy group of both dietary and synthetic benzodioxole compounds.[38]

Cytotoxic effects associated with their long-term use however have limited any therapeutic benefits of catechol and benzodioxole-based pharmacophores. Catecholic therapeutics and their oxidative metabolites cause damage to biomolecules by oxidative stress. There is therefore a need for effective antioxidant therapeutic interventions for drugs that contain this pharmacophore.

Thus, the present study explores the synthesis of novel isoindoline based-catecholamines and benzodioxoles, as well as their nitroxide derivatives (**Figure 5.3**). As the first examples of catechol-based nitroxides, the target compounds could be explored as potential dual antioxidant therapeutics as well as synthetic templates/precursors for building functional materials. The target isoindoline-based catecholamines such as compound **154** could potentially serve as a new type of sympathomimetic amphetamine mimicking drug owing to their structural similarities

to potent therapeutics like L-Dopa **147**, dopamine **152** and adrenaline **319**. L-Dopa **147** for instance is identified as the standard-of-care treatment for Parkinson's disease.

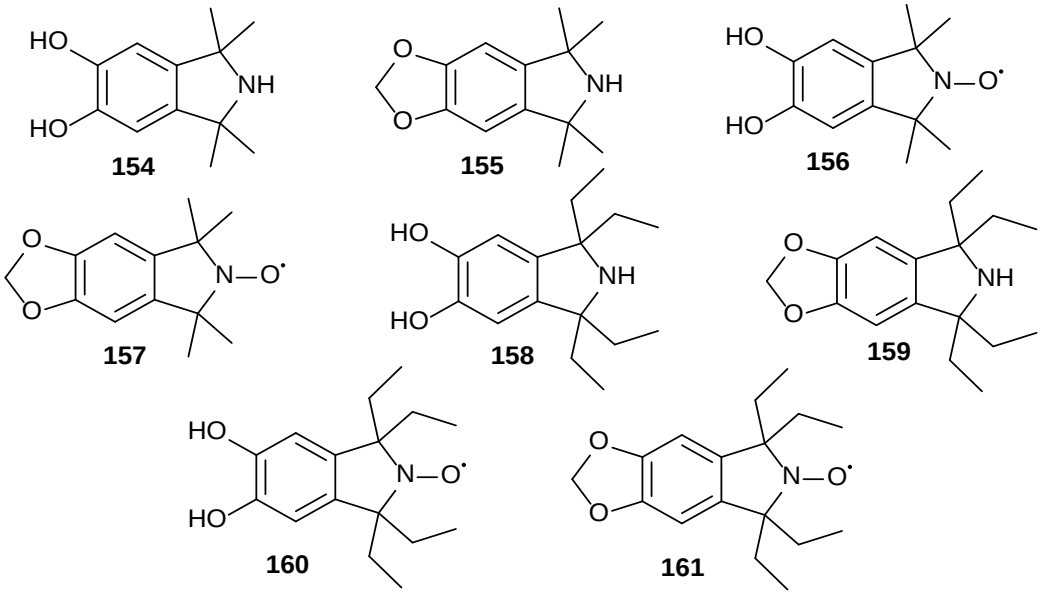


Figure 5.3. Target catechol-based compounds: Catecholamines TMI **154** and TEI **158**, catechol TMIO **156** and TEIO **160**, benzodioxole TMI **155** and TEI **159**, benzodioxole TMIO **157** and TEIO **161**.

Compound **154** could also be used as a building block for catechol-containing functional materials via synthetic modifications through either the catechol or the amino functional group. Along with their nitroxide counterparts (**156** and **160**), the target catecholamines (**154** and **158**) are also designed as potential substrates for building new nanocomposite drug delivery systems. In addition, other nanocomposite materials could be built from these hybrid compounds for potential applications in sensing and electronic devices, catalysis and (bio)chemical reactions.

The catechol-nitroxide analogues (**156** and **160**) are also designed as potential dual action antioxidants therapeutics owing to the presence of both the catechol and the free radical moieties. It is anticipated that the isoindoline based benzodioxole target compounds (**155**, **157**, **159** and **161**) may display similar sympathomemitic efficacies to their parent templates (**328** and **153**). In addition to the potential sympathomemitic effects, the nitroxide derivatives (compounds **157** and **161**) could also reduce both

drug-induced and disease related oxidative stress through their reactive oxygen scavenging activities.

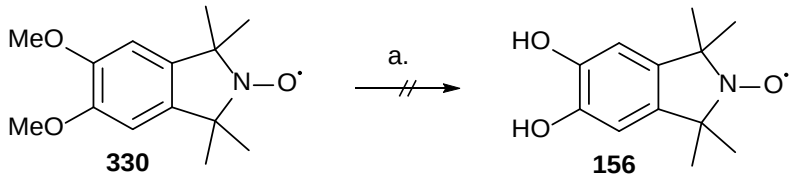
Since increasing the steric bulk at the α-carbon slows the rate with which the nitroxide moiety gets reduced in biological system, each class of target compounds consists of the tetramethyl and tetraethyl-substituted isoindoline-based catecholic compounds. The syntheses are discussed in the subsequent sections.

5.2 Results and Discussion

5.2.1 Synthesis of Tetramethyl Isoindoline Analogues of Catecholamine, Benzodioxole and their Corresponding Nitroxides.

5.2.1.1 Attempted Synthesis of Catechol TMIO 143 via Boron Tribromide Demethylation Protocol

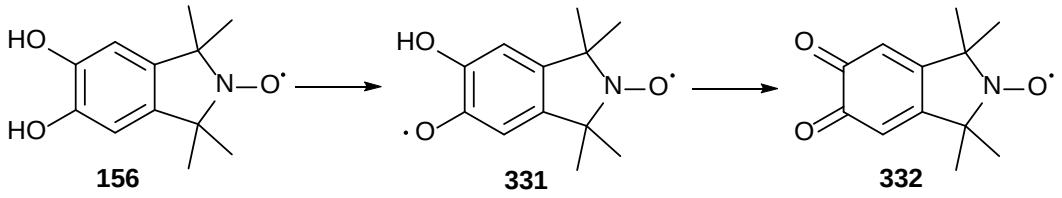
As outlined in **Scheme 5.4** below, previous attempts to synthesize catechol TMIO **156** from the dimethoxy TMIO precursor **330** were undertaken using boron tribromide as the demethylating reagent.[39] Unfortunately, a complex mixture of products was obtained under these conditions.



Scheme 5.4. Attempted synthesis of catechol TMIO **156** via boron tribromide demethylation of dimethoxy TMIO precursor **330**. Reagents and conditions: a. BBr_3, DCM, -78 ^oC.

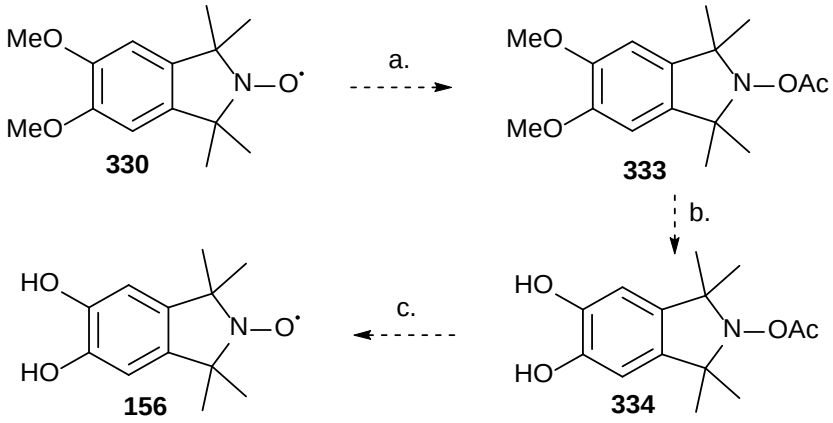
This was attributed to a nitroxide-mediated degradation of the desired catechol TMIO **156** to the highly reactive and unstable semiquinone **331** and quinone **332** derivatives as outlined in **Scheme 5.5** below. Evidence to support the formation of these oxidative by-products was obtained by the presence of a strong C=O stretch (characteristic of quinones) at 1694 cm^{-1} in the FTIR spectrum of the isolated product mixture. Although such proposed nitroxide-mediated degradation of **156** seems

plausible, one should not ignore the role of the boron tribromide reagent into the decomposition pathway.



Scheme 5.5. Proposed nitroxide mediated oxidative decomposition of catechol **156** to semiquinone **331** and quinone **332**.

As mentioned in **Section 2.12** (**Chapter 2**), the nitroxide moiety could potentially complex with the boron reagent to generate other reactive radical species that may facilitate the degradation process. To avoid such proposed nitroxide-mediated decomposition of the target catechol TMIO **156**, the initial alternative approach employed here (in the synthesis of **156**) was to first protect the nitroxide moiety of dimethoxy precursor **330** prior to the demethylation step. As outlined in **Scheme 5.6** below, by protecting the nitroxide **330** with an acetyl-protecting group, it was envisaged that this might allow the demethylation to be carried out smoothly with no associated nitroxide-mediated decomposition of the catechol substrate.

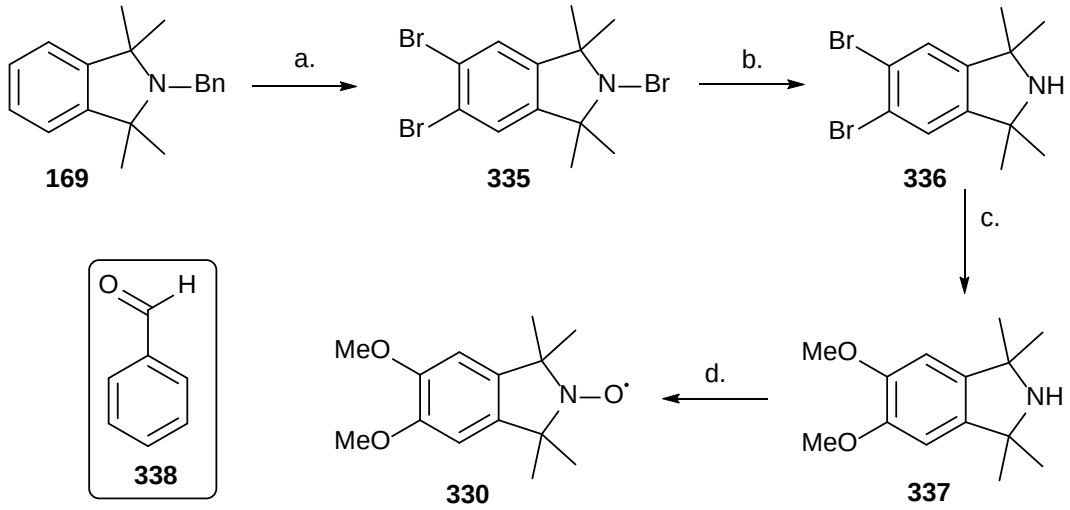


Scheme 5.6. Proposed synthesis of catechol TMIO **156** via *N*-acetoxy protecting group strategy. Reagents and conditions: a. Pd/C, H_2, TEA, AcCl, THF, 0 ^oC; b. BBr_3, DCM, -78 ^oC; c. NaOH, MeOH/H_2O.

The next section discusses the synthesis and *N*-acetoxy protection of **330** and the subsequent demethylation of the resulting acetyl protected intermediate **333**.

5.2.1.2 Synthesis of 5,6-Dimethoxy TMIO 330

The dimethoxy nitroxide precursor **330** was readily synthesized in five steps from the tetramethylated Grignard reaction product **169** by following previous methodologies (with slight modifications) developed in the Bottle group at QUT (**Scheme 5.7**).[40]



Scheme 5.7. Synthesis of dimethoxy TMIO **330**. Reagents and conditions: a. Br_2, $AlCl_3$, DCM, 0 ^oC-RT, O/N, 89%; b. H_2O_2, $NaHCO_3$, MeOH/DCM, 10 min, 100%; c. 5 M NaOMe/MeOH, CuI, DMF, reflux, 1 d, 88%; d. *m*CPBA, DCM, 0 ^oC-RT, 2 h, 87%.

The starting compound **169** was readily obtained in two steps from commercially available phthalic anhydride **167** as discussed in **Chapter 2** (**Section 2.2.1.1**). When compound **169** was treated with a seven-fold excess of bromine in the presence anhydrous aluminium trichloride and pyridine, the crude residue obtained was triturated with methanol to give the tribromo compound **335** in 90% yield. The trituration step ensured complete separation of compound **335** from the benzaldehyde side-product **338** formed during the bromine-facilitated oxidative debenzylation of **169**. Compound **335** was then reduced quantitatively to the corresponding dibromoamine **336** by reacting with aqueous hydrogen peroxide in the presence of sodium bicarbonate. In the literature procedure for the synthesis of **336**, the

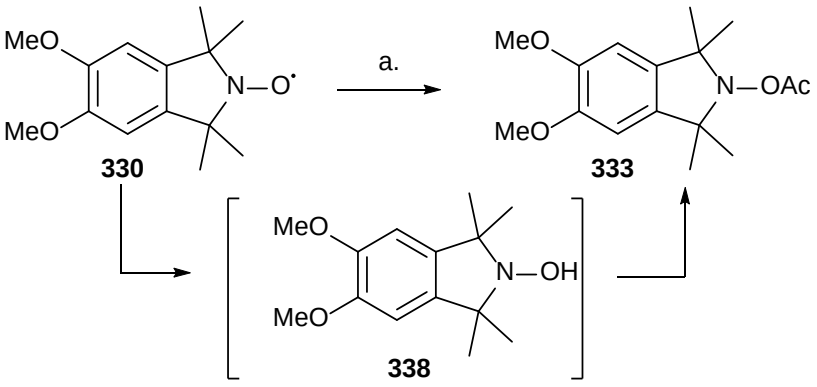
tribromide intermediate **335** was not isolated. Instead, the crude mixture (of tribromide **335** and benzaldehyde **338**) was reduced to give a mixture of the desired dibromoamine **336** and the benzaldehyde **338** side-product. Pure compound **336** was then obtained via successive acid-base extractions of the crude mixture which ensured complete removal of the benzaldehyde **338**. For optimum yield however, several backwashes of the benzaldehyde extracts with sulfuric acid were required. Ultimately, purification of **335** by trituration and subsequent reduction to the dibromoamine **336** was an equally efficient protocol to furnish **336** that avoided the use of large volumes of acid and base (and the neutralization step).

For the synthesis of dimethoxyamine **337**, dibromoamine **336** was subjected to the copper-catalyzed nucleophilic substitution of aryl halides with an alkoxide as discussed is **Section 2.2.1.3**. The methanolysis protocol involved refluxing the aryl halide in a 25 mol% sodium methoxide solution in the presence of catalytic copper (I) iodide and DMF as cosolvent. The dimethoxy derivative **337** was obtained in 88% yield and was used in the subsequent step without further purification. Compound **337** was then converted to the nitroxide derivative **330** under a mild *m*CPBA oxidation conditions in 87% yield. All characterization data obtained for compounds **330** and **335-327** were consistent with those reported in the literature.[39,40]

5.2.1.3 *N*-Acetyl Protection of Dimethoxy TMIO 330 and Subsequent Attempted Boron Tribromide Demethylation

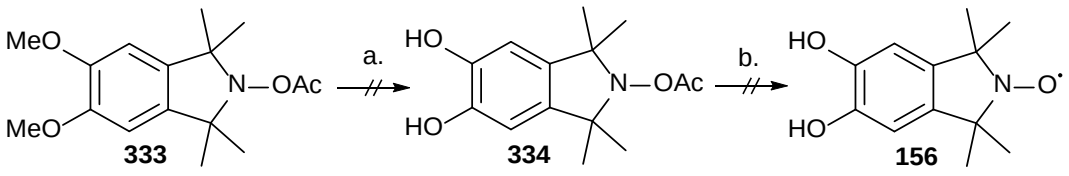
Due to the ease of introduction and removal, the acetyl protecting group strategy is a convenient way to mask a nitroxide moiety. Moreover, the protection and deprotection steps are generally quantitative transformations. In the synthesis of compound **333**, the nitroxide precursor **330** was first hydrogenated to give the hydroxylamine **339** (**Scheme 5.8**). The hydroxylamine intermediate **339** was then allowed to react *in situ* with acetyl chloride in the presence of triethylamine for two hours. The desired *N*-acetoxy dimethoxy compound **333** was obtained in 95% yield following purification of the crude product by silica gel column chromatography. The most diagnostic feature in the ^{1}H NMR spectrum of **333** was the characteristic *N*-acetoxy protons that were observed as a singlet at 2.2 ppm (3 H). The two singlets at 1.38 and 1.46 ppm, each integrating for 6 protons corresponds to the two sets of

CH_3 groups. The non-equivalence observed for the two sets of CH_3 groups arises from a combination of a slow nitrogen inversion on the NMR time scale. The equivalent dimethoxy and aryl protons were also observed as singlets at 3.88 (6 H) and 6.61 ppm (2 H) respectively. The strong C=O stretching frequency observed at 1753 cm^{-1} in the FTIR spectrum of **333** was consistent with the presence of an acetyl functionality. Compound **333** was >95% pure by HPLC analysis and its melting range was determined to be 84-85 ^{o}C.



Scheme 5.8. Synthesis of *N*-acetoxy protected dimethoxyamine **333**. Reagents and conditions: a. Pd/C, H_2, TEA, AcCl, THF, 0 ^{o}C-RT, 2 h, 95%.

Having successfully synthesized the *N*-acetoxy protected dimethoxy derivative **333**, the next step was to deprotect the dimethoxy group using mild boron tribromide demethylation conditions to furnish the corresponding *N*-acetoxy catechol **334** (**Scheme 5.9**). Upon treating **333** with three molar equivalents of boron tribromide, TLC analysis of the reaction mixture indicated complete consumption of the starting material after one day.



Scheme 5.9. Attempted boron tribromide demethylation of *N*-acetoxy protected dimethoxyamine **333**. Reagents and conditions: a. BBr_3, DCM, -78 ^{o}C-RT, 1 d; b. 1 M NaOH, MeOH/H_2O, 0 ^{o}C-RT, 4 h.

^{1}H NMR analysis of the crude product indicated the loss of the singlet corresponding to the dimethoxy protons at 3.88 ppm. This, evidently, supported the successful demethylation of compound **333**. However, no phenolic proton signal was observed in the ^{1}H NMR spectrum of the isolated crude product. This could be attributed to a likely complexation between the catechol moiety of compound **334** and the boron tribromide. Like most trisubstituted boron centres, the boron atom of boron tribromide is sp^2 hybridized and has a trigonal planar geometry and an empty p orbital. The presence of the empty p orbital gives boron tribromide its weak Lewis acid characteristics. This therefore allows boron tribromide to be susceptible to nucleophilic attack by a variety of nucleophiles including hydroxide, cyanide, fluoride, α-hydroxy-carboxylic acids, dicarboxylic acids, and diols. Under appropriate conditions, trisubstituted boron compounds such as boronic acids can rapidly react with catechols to form stable boronic esters.[41,42] The resulting cycloboronates have tetrahedral geometry with an sp^3 hybridization at the boron centre.

Attempt to purify the crude product isolated from the boron tribromide demethylation reaction by silica gel chromatography were unsuccessful as no product was recovered from the column even when more polar eluents (DCM/MeOH) were used. In this case, it is likely that the catechol moiety of **334** formed a complex with the silica gel particles. Also, purification of the crude product by trituration or recrystallization was unsuccessful. Therefore, the crude product was subsequently hydrolyzed to the corresponding nitroxide **156** under basic conditions. Unfortunately, a complex mixture of products was isolated from the hydrolysis reaction as verified by TLC. In the FTIR spectrum of the hydrolysis product mixture, a strong C=O absorption was observed at 1740 cm^{-1}. This suggested the likely decomposition of the desired product **156** to the highly reactive quinone derivative **332**. Also, no characteristic HRMS signal could be obtained for the crude mixture. This suggested that the target catechol TMIO **156** could have undergone an oxidative self-polymerisation to give polyquinoline derivatives under such basic hydrolysis conditions.

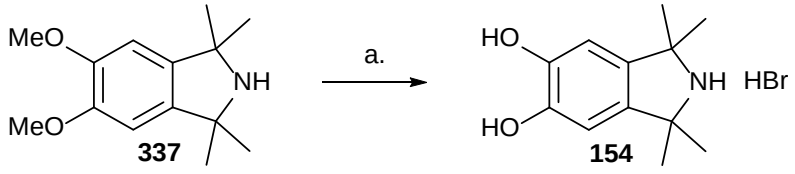
Thus, due to its high reactivity and redox characteristic, the target catechol TMIO **156** could not be isolated following the attempted boron tribromide demethylation of

333 and the subsequent hydrolysis reaction. As a result of the likely complexation and redox nature of the target catechol **156**, any alternative demethylation conditions should preferably exclude the use of Lewis acid reagents. The following sections discuss the synthesis of catecholamine TMI compound **154** and its catechol nitroxide **156** as well as the methylenedioxy derivatives (**155** and **157**) via demethylation of the dimethoxyamine precursor **337** under Bronsted acid conditions.

5.2.1.4 Synthesis of Catecholamine TMI 154

Besides Lewis acids, Bronsted acids are the other frequently used ether cleavage reagents. The most common Bronsted acids employed include hydrogen halides such as hydrochloric and hydrobromic acids. The demethylation protocol generally involves refluxing the ether substrate in concentrated Bronsted acid solutions. Such harsh reaction conditions were, however, not attempted on the acetyl protected dimethoxy substrate **333** or the nitroxide precursor **330**. This was due to the likely hydrolysis of the acetyl protecting group of **333** and subsequent acid-catalyzed decomposition of the nitroxide intermediate **330**. However, Bronsted acid reagents could readily be employed in the synthesis of the target catecholamine TMI compound **154** via demethylation of the dimethoxyamine precursor **337**. Successfully synthesis of catecholamine TMI **154** may provide an alternative route to access the target catechol TMIO **156** via a mild peroxy acid oxidation of the catecholamine **154**. In addition, catecholamine **154** could also serve as a precursor to target methylenedioxy TMI **155** and TMIO **157** compounds.

Thus when dimethoxyamine **337** was refluxed in concentrated hydrobromic acid solution, complete demethylation was achieved after twelve hours (**Scheme 5.10**). The target catecholamine **154**, isolated as the hydrobromide salt, was obtained as a grey precipitate in 84% yield.

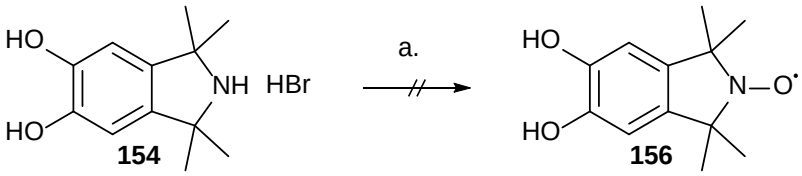


Scheme 5.10. Synthesis of catecholamine TMI **154**. Reagents and conditions: a. HBr (45% wt./v in H_2O), reflux, O/N, 84%.

Evidence to support this transformation was obtained from the loss of the dimethoxy protons signal at 3.88 ppm in the ^{1}H NMR spectrum of **154**. The equivalent aryl and diphenolic protons signals of **154** were also observed as singlets at 6.63 and 9.23 ppm respectively. In the FTIR spectrum of **154**, a characteristic strong broad OH stretch observed at 3147 cm^{-1} also supported the presence of phenol functionality. The identity of **154** was further confirmed by the presence of the molecular ion of the protonated amine at m/z 202 in the HRMS spectrum. Catecholamine **154** decomposed at temperatures above 331-333 ^oC.

5.2.1.5 Synthesis of Catechol TMIO 156 from Catecholamine TMI 154

With the catecholamine derivative **154** in hand, synthesis of the catechol TMIO **156** was then attempted under mild peroxy acid oxidation conditions. However, upon subjecting compound **154** to sodium tungstate-hydrogen peroxide oxidation conditions (**Scheme 5.11**), FTIR analysis of the isolated product indicated the presence of a characteristic strong C=O stretching absorption at 1740 cm^{-1}. In addition, no characteristic OH stretch was observed above 3000 cm^{-1} in the FTIR spectrum.



Scheme 5.11. Attempted synthesis of catechol TMIO **156** via peroxy acid oxidation of catecholamine TMI **154**. Reagents and conditions: a. H_2O_2, H_2O/MeOH, RT; or *m*CPBA, DCM/MeOH.

This, evidently, indicated the target catechol TMIO **156** was likely oxidized to the quinone derivative under such oxidation conditions. The reddish-brown appearance of the isolated product is a further characteristic indication of the presence of a quinone **332** intermediate. Also, no characteristic mass was obtained from the HRMS

analysis. A similar result was also obtained when *m*CPBA was used in the place of hydrogen peroxide.

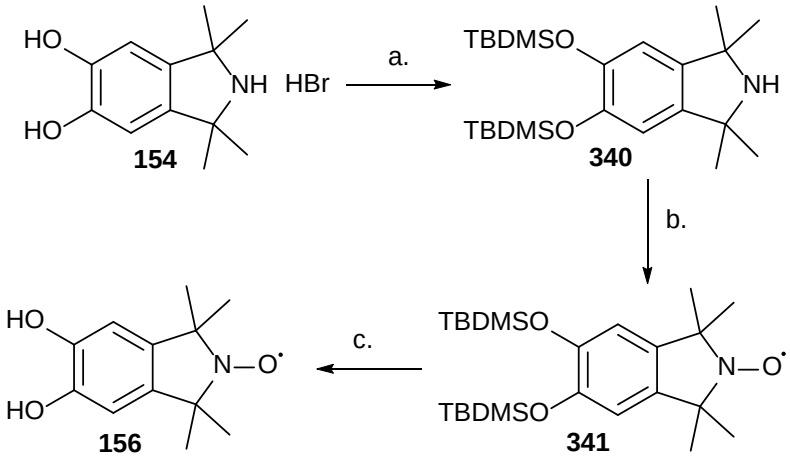
At this stage, it was difficult to determine whether such oxidative decomposition of the preformed-catechol TMIO **156** to the quinone derivative **332** was primarily a spontaneous process (autoxidation facilitated by both catechol and the free radical moieties), or facilitated by the hydrogen peroxide (or *m*CPBA) as the active oxidizing agent, or a combination of both.

In a final attempt to synthesize compound **156**, it was thought necessary to protect the catechol moiety of catecholamine **154** prior to attempting the oxidation step. This may ensure smooth oxidation of the catechol-protected amine to the corresponding nitroxide which in turn could be deprotected to give the desired catechol TMIO **156**. However, since the oxidation of catechols to quinones usually occurs under both oxidizing and basic conditions, the choice of protecting group herein should exclude protecting groups that are removed under such reaction conditions (oxidizing conditions or conditions above pH 8). Since silyl ether protecting groups are commonly removed under neutral or mild acidic conditions, *t*-butyldimethylsilyl group (TBDMS) was the protecting group of choice used in this case.

As outlined in **Scheme 5.12** below, silylation of **154** was achieved by allowing it to react with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in the presence of excess imidazole for four days. The silyl ether **340** was determined to be slightly unstable when the crude isolated product was subjected to standard column chromatography purification. Attempts to purify the isolated crude silyl ether **340** by silica gel chromatography produced a complex mixture of desilylated products as verified by ^{1}H NMR spectroscopy. However, after several mild aqueous base washes with 0.5 M sodium hydroxide solution, compound **340** was of sufficient purity by ^{1}H NMR spectroscopy and was used in the subsequent step without further purification.

The loss of the characteristic diphenol protons signal (singlet at 9.23 ppm) in the ^{1}H NMR spectrum of **340** evidently indicated successful transformation. The protons of the di-*tert*-butyldimethylsilyl group were also observed as singlets at 0.19 (12 H) and 0.99 ppm (18 H). HRMS analysis further confirmed the identity of **340** by the presence of the molecular ion plus proton $[M + H]^{+}$ mass at m/z 436. The FTIR

spectrum of **340** also confirmed the loss of a strong broad OH stretching frequency at 3147 cm^{-1} as well as the presence of a weak NH absorption at 3100 cm^{-1}.



Scheme 5.12. Synthesis of catechol TMIO **156** via TBDMS protection of catecholamine TMI **154**. Reagents and conditions: a. TBDMSCl, imidazole, DMF, 4 d, 64%; b. *m*CPBA, DCM, 2 h, 83%; c. TBAF, THF, 30 min, 79%.

The aminosilyl ether **340** was then converted to the corresponding nitroxide **341** under mild *m*CPBA oxidation conditions. To avoid potential acid-catalyzed cleavage of the silyl ether-protecting group by the resulting *m*-chlorobenzoic acid by-product, the oxidation of **340** was carried out in the presence of sodium hydrogen carbonate. The silyl-protected nitroxide **341** was obtained as a yellow solid in 83% yield. The identity of **341** was confirmed by the presence of the $[M + H]^+$ and $[M + Na]^+$ ions at m/z 436 and 450 respectively in the HRMS spectrum. The melting range determined for the isolated product was 142-143 °C.

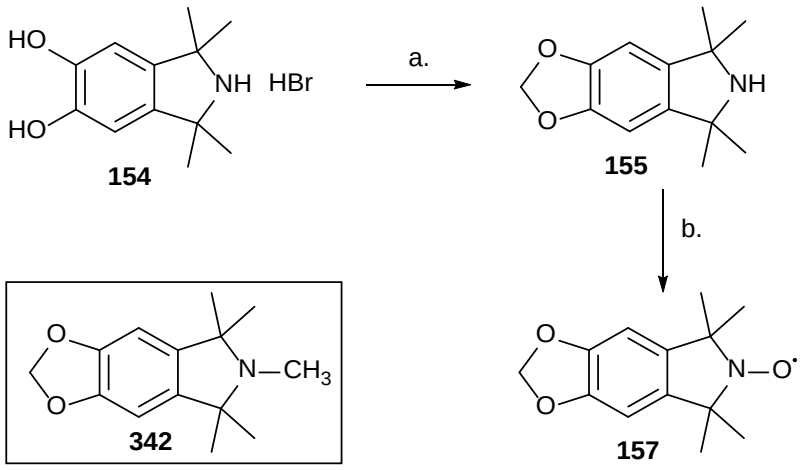
Subsequent deprotection of the silyl ether **341** was carried out with a one molar solution of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran. As anticipated, the deprotection proceeded smoothly in 30 minutes to afford target catechol TMIO **156** as a yellow solid in 79% yield. The identity of **156** was confirmed by HRMS analysis with the $[M + H]^+$ ion observed at m/z 223. A strong broad OH stretching frequency for the diphenol group was observed at 3055 cm^{-1} in the FTIR spectrum. The melting range determined for **156** was 225-227 °C (decomposed).

The above results thus indicated that the likely decomposition of catechol TMIO **156** to the quinone derivative **322** proposed in the preceding sections is not primarily a nitroxide-mediated process. Instead, it appears to be primarily facilitated by the presence of an oxidizing agent (*m*CPBA or H_2O_2), a base (NaOH) or via the formation of stable catecholborane complexes with BBr_3. Furthermore, no C=O stretching frequencing was observed in the FTIR spectrum of **156**. This thus indicated that compound **156** is fairly stable and does not spontaneously decompose to generate the highly reactive quinone oxidative by-product **332** as was initially proposed.

With the targets catecholamine TMI **154** and catechol TMIO **156** in hand, the next section discusses the synthesis of the tetramethyl-substituted methylenedioxy derivatives **155** and **157**.

5.2.1.6 Synthesis of Benzodioxole TMI 155 and TMIO 157

Methylenation of catechols to the corresponding methylenedioxy groups is commonly performed with methylene halides as the methylenating reagent in the presence of a base.[43,44] The reaction is generally carried out in a refluxing dipolar aprotic solvent such as dimethylformamide. **Scheme 5.13** below outlines the synthetic route undertaken to furnish methylenedioxy TMI **155** and the corresponding nitroxide **157**.



Scheme 5.13. Synthesis of benzodioxole TMI **155** and TMIO **157**. Reagents and conditions: a. DCM, NaOH, DMSO, reflux, 2 h, 71%; b. *m*CPBA, DCM, 2 h, 89%.

Initially, when a solution of catecholamine **154** and two equivalents of dibromomethane in DMSO was refluxed for two hours in the presence of sodium hydroxide, the product isolated was predominantly the *N*-methyl methylenedioxy derivative **342**, as confirmed by HRMS, ^{1}H and ^{13}C NMR spectroscopies. When the reaction was carried out with excess dichloromethane in the place of dibromomethane, an almost equimolar mixture of the target methylenedioxy TMI **155** and the *N*-methyl derivative **342** was obtained as verified by ^{1}H NMR analysis.

Although there are examples in the literature for the reaction of dichloromethane with secondary amines under similar basic conditions, the products isolated in those cases were the *N*-(chloromethyl) derivatives (no *N*-methyl derivative was isolated).[45-47] The mechanism for the formation of the unexpected *N*-methyl derivative **341** herein is at this stage elusive and was not further investigated because optimum conditions were established for the exclusive formation of the desired methylenedioxy TMI **155**. The optimum condition for the synthesis of methylenedioxy TMI **155** required the use of 2.5 molar equivalents of dichloromethane and a two-hour reflux. Under these conditions, the target methylenedioxy TMI **155** was obtained in 71% yield following purification by column chromatography. The identity of compound **155** was confirmed by FTIR, HRMS, ^{1}H and ^{13}C NMR spectroscopies. In the ^{1}H NMR spectrum of **155**, the methylenedioxy group protons were shown as a singlet at 6.55 ppm (2 H). The mass of the $[M + H]^{+}$ ion was also observed at m/z 220 in the HRMS spectrum of **155**. Compound **155** was determined to be >95% pure by HPLC analysis.

Subsequent oxidation of **155** to the corresponding nitroxide **157** was achieved under mild *m*CPBA conditions. Compound **157** was obtained as a yellow solid in 89% yield after purification by silica gel column chromatography. The structure of **157** was also confirmed by the presence of the $[M + 2H]^{+}$ and $[M + Na]^{+}$ ions at m/z 236 and 257 respectively in the HRMS spectrum. Compound **157** was >95% pure by HPLC analysis.

Having established the optimum conditions for the synthesis of the tetramethyl-substituted target catechols (**154** and **156**) and methylenedioxy compounds (**255** and

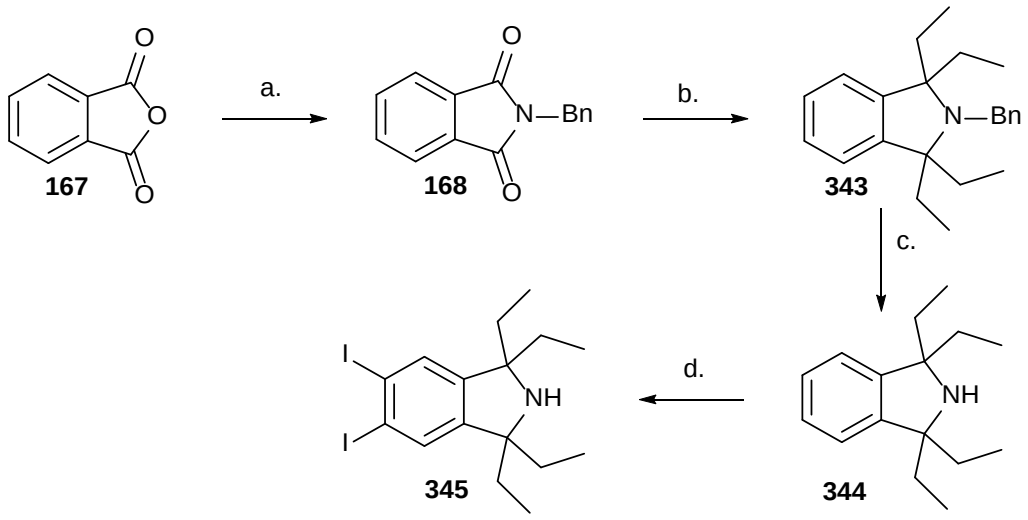
157), the next section details the use of similar conditions for the synthesis of the tetraethyl-substituted counterparts (**158-161**).

5.2.2 Synthesis of Tetraethyl-substituted Isoindoline Analogues of Catecholamine, Benzodioxole and their Corresponding Nitroxides.

As noted in the introductory chapter, the stability of the nitroxide moiety partly depends on steric effects at the carbon α to the radical centre. This, therefore, makes tetraethyl-substituted nitroxides reduced at a much slower rate than their tetramethyl counterparts under biological conditions. Thus, synthesising the tetraethyl versions of catecholamine and the corresponding nitroxide compounds may provide robust antioxidant systems as well as provide more insights into the structural-activity relationship when compared to the tetramethyl counterparts.

5.2.2.1 Synthesis of 5,6-Diiodo-TEI 335

5,6-Diiodo-1,1,3,3-tetraethylisoindoline **335** was synthesized by following literature procedures as outlined in **Scheme 5.14** below.[48,49] The benzyl phthalimide **168** was generated by refluxing commercially available phthalic anhydride **167** and benzylamine in acetic acid.



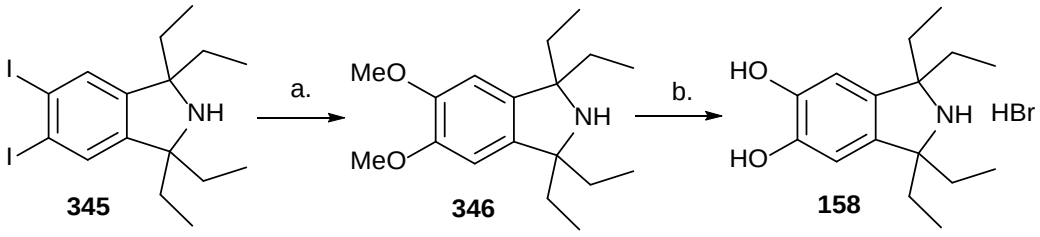
Scheme 5.14. Synthesis of diiodo TEI **345**. Reagents and conditions: a. $BnNH_2$, AcOH, reflux, 1 h, 98%; b. EtMgI, Et_2O, toluene, reflux, 4 h, 38%; c. H_2, Pd/C, 50 psi, 3 h, 94%; d. H_5IO_6, KI, conc. H_2SO_4, 0 °C to RT, 3 h, 80%.

Exhaustive alkylation of compound **168** with a sixfold excess of freshly prepared ethylmagnesium Grignard furnished the tetraethylated product **343** in modest yield of 38%. Subsequent palladium catalyzed hydrogenolysis of the Grignard reaction product **343** furnished the debenzylated secondary amine **344** almost quantitatively.

Allowing **344** to react with periodic acid and potassium iodide in cold concentrated sulfuric acid facilitated the successful di-iodination of compound **344**. The periodic acid and potassium iodide reagents act as a source of a strongly electrophilic iodonium ion known to facilitate the iodination.[49] Compound **345** was obtained in 80% yield following purification by silica gel chromatography. All characteristic data obtained were consistent with the literature.[49]

5.2.2.2 Synthesis of Catecholamine TEI 158

Diiodo TEI **345** was subjected to similar copper-catalyzed methanolysis conditions employed for the synthesis of **337** (**Scheme 5.7**) as outlined in **Scheme 5.15** below. The 5,6-dimethoxyamine derivative **346** was obtained in 82% yield following purification by silica gel flash column chromatography. The identity of compound **346** was confirmed by FTIR, HRMS, ^{1}H and ^{13}C NMR spectroscopy. The dimethoxy protons were observed as a singlet at 3.87 ppm (6 H) while the $[M + H]^{+}$ ion was observed at m/z 192 in the ^{1}H NMR and HRMS spectra of **346** respectively. Compound **346** was >95% pure by HPLC analysis and its melting range was determined to be 58-59 ^{o}C.



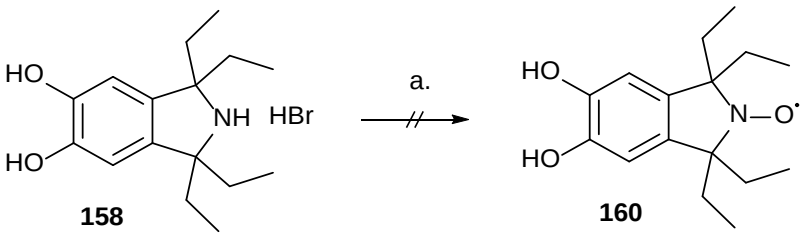
Scheme 5.15. Synthesis of catecholamine TEI **158**. Reagents and conditions: a. NaOMe, CuI, DMF, reflux, 1 d, 82%; b. HBr (45% wt./v in H_2O), reflux, O/N, 83%.

Demethylation of **346** to the corresponding catecholamine derivative **158** was achieved under refluxing conditions with concentrated aqueous hydrobromic acid solution (48% in water). The target catecholamine **158** was obtained as the

hydrobromide salt as a light grey precipitate in 83% yield. The loss of the singlet at 3.87 ppm for the dimethoxy group protons of **346** and the appearance of the diphenol protons signal as singlet at 9.23 ppm in the ^{1}H NMR spectrum of **158** were supporting evidence for successful demethylation of **346**. Further evidence was obtained by the presence of a characteristic strong broad OH absorption at 3343 cm^{-1} in the FTIR spectrum of **158**. The mass of the molecular ion $[M]^+$ was observed at m/z 264 in the HRMS spectrum of **158**. Compound **158** decomposed at temperatures above 251-252 ^{o}C.

5.2.2.3 Synthesis of Catechol TEIO 160

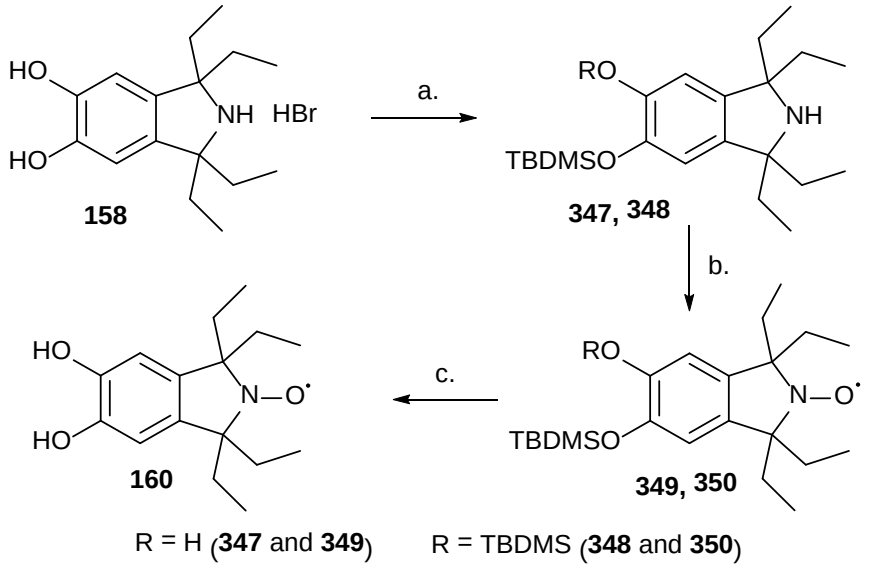
Like the tetramethyl counterpart **154**, attempted oxidation of the tetraethyl-substituted catecholamine **158** with either *m*CPBA or hydrogen peroxide (**Scheme 5.16**) resulted in the formation of oxidative quinone by-products similar to those previously observed, as verified by FTIR spectroscopy. The isolated product had similar quinone characteristics with a C=O absorption at 1750 cm^{-1} in the FTIR spectrum.



Scheme 5.16. Attempted synthesis of catechol TEIO **160** via peroxy acid oxidation of catecholamine TEI **158**. Reagents and conditions: a. H_2O_2, H_2O/MeOH, RT; or *m*CPBA, DCM/MeOH.

Thus, the successful synthesis of catechol TEIO **160** could only be accomplished via the silyl ether protecting group strategy as outlined in **Scheme 5.17** below. When the catecholamine **158** was reacted with *tert*-butyldimethylsilyl chloride in the presence of imidazole, the product isolated was a mixture of the mono- and di-silyl protected ethers (**347** and **348**) as verified by ^{1}H NMR analysis. Although subsequent oxidation of the mixture (**347** and **348**) to the corresponding nitroxides (**349** and **350**) proceeded smoothly under mild *m*CPBA conditions, a reasonably pure di-silyl

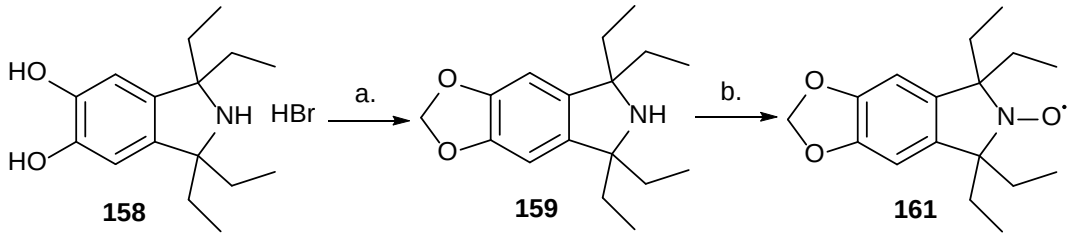
protected ether **348** was obtained when the crude product mixture (of **347** and **348**) was washed with a dilute 0.5 M sodium hydroxide solution. The silyl ether nitroxide mixture (**349** and **350**) was then deprotected with a 1 M tetrabutylammonium fluoride solution to give the desired catechol TEIO **160**. Compound **160** was obtained as a bright orange solid in 67% yield over the three steps from **158**. A strong OH stretch was observed at 3354 cm^{-1} in the FTIR spectrum of **160**. The $[M + H]^+$ and $[M + Na]^+$ ions were observed at m/z 279 and 301 respectively in the HRMS spectrum. The recorded melting range for **160** was 150-151 °C.



Scheme 5.17. Synthesis of catechol TEIO **160** via TBDMS protection of catecholamine TEI **158**. Reagents and conditions: a. TBDMSCl, imidazole, DMF, 4 d; b. *m*CPBA, DCM, 2 h; c. TBAF, THF, 30 min, 67% (over the 3 steps).

5.2.2.4 Synthesis of Benzodioxole TEI 159 and TEIO 161

The tetraethyl methylenedioxy derivatives (**159** and **161**) were readily obtained from catecholamine precursor **158** as depicted in **Scheme 5.18** below. When a solution of **158** and three molar equivalents of dichloromethane in DMSO was refluxed for three hours, the crude residue obtained was purified by column chromatography to give methylenedioxy TEI **159** in 68% yield. The most diagnostic feature as a supporting evidence for this transformation was the presence of a singlet at 6.05 ppm in the ^1H NMR spectrum of **159**. This was consistent with the presence of the characteristic methylenedioxy proton (CH_2) signal.



Scheme 5.18. Synthesis of benzodioxole TEI **159** and TEIO **161**. Reagents and conditions: a. DCM, NaOH, DMSO, reflux, 2 h, 68%; b. *m*CPBA, DCM, 2 h, 85%.

Also, neither a phenolic proton signal nor its characteristic OH absorption was observed in the ^{1}H NMR and FTIR spectra of **159** respectively. The extra methylenedioxy carbon was also observed at 69 ppm in the ^{13}C NMR spectrum. The $[M + H]^{+}$ ion for compound **159** was observed at m/z 276 in the HRMS spectrum and **159** was >95% pure by HPLC. Subsequent oxidation of **146** to the corresponding nitroxide **161** was facilitated by *m*CPBA. Catechol TEIO **161** was obtained as a yellow solid in 85% yield and its identity was verified by FTIR and HRMS analysis.

5.3 Summary of Results

The synthesis of a series of isoindoline-based catecholamine and methylenedioxy compounds (**154-161**) was described. The target compounds comprised of both the tetramethyl and the tetraethyl-substituted secondary amines and the corresponding nitroxides. Notably, the target compounds are the first examples of catechol-based nitroxide compounds synthesized. The target catecholamine derivatives (**154** and **158**) were readily obtained by demethylation of the dimethoxyamine precursors (**337** and **345**) under refluxing Bronsted acid conditions with concentrated hydrobromic acid.

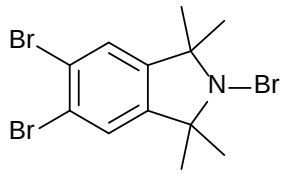
Initial attempts to synthesize the catechol nitroxides (**156** and **160**) via either boron tribromide demethylation of the *N*-acetoxy protected dimethoxy derivative **333** or direct peroxy acid oxidation of the catecholamine derivatives (**154** and **158**) resulting in the formation of presumably the highly reactive quinone derivatives as verified by FTIR spectroscopy. The target catechol nitroxides (**156** and **160**) were however obtained via a silyl ether protection of the catecholamines (**154** and **158**) followed by oxidation to the corresponding silyl ether nitroxides (**341**, **349** and **350**) which were

in turn readily deprotected with TBAF to furnish the desired catechol nitroxides (**156** and **160**).

The methylenedioxyamine derivatives (**155** and **159**) were prepared by base-catalyzed methylation of catecholamines (**154** and **158**) with dichloromethane. Although the *N*-methyl methylenedioxyamine derivative **342** was initially obtained under the base-catalyzed methylation conditions (with dibromomethane or excess DCM), optimum conditions for the synthesis of the desired methylenedioxyamine compounds (**155** and **159**) required the use of 2.5-3 molar equivalents of DCM and a refluxing time of three hours or less. The methylenedioxy nitroxides (**157** and **161**) were readily obtained by a mild *m*CPBA oxidation of the methylenedioxyamine derivatives (**155** and **159**).

5.4 Experimental

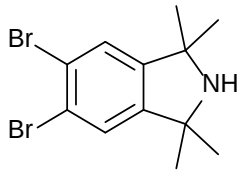
5.4.1. Synthesis of 2,5,6-Tribromo-1,1,3,3-tetramethyl isoindoline 335



Bromine (2.7 mL, 52.75 mmol, 7 equiv.) was added dropwise to a solution of 2-benzyl-1,1,3,3-tetramethylisoindoline **169** (2 g, 7.54 mmol, 1.00 equiv.) and pyridine (200 µL, 2.49 mmol, 0.33 equiv.) in DCM (40 mL) at 0 °C under an Ar atmosphere. The reaction mixture was stirred for 15 min followed by the addition of anhydrous $AlCl_3$ (3 g, 22.61 mmol, 3.00 equiv.). Stirring was continued overnight while allowing the reaction mixture to return to RT. The reaction was cooled in ice bath and then basified with 10 M NaOH (25 mL) and stirred vigorously (10 min) until the solution was almost clear. The mixture was extracted with DCM (70 mL x 4) and the combined DCM extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give yellowish brown oil. The crude residue was then purified by triturating with methanol to give 2,5,6-tribromo-1,1,3,3-tetramethylisoindoline **335** as a pale yellow solid (2.76 g, 89%). Mp. 109-110 ^{o}C (Lit.,[50] 110–112 ^{o}C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.42 (s, 12 H, CH_3), 7.39 (s, 2

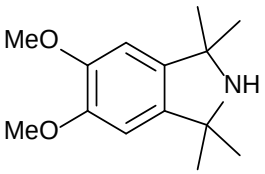
H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 28.0 (C-*C*H_3), 69.3 (*C*-CH_3), 123.2 (Ar-*C*), 127.1 (Ar-*C*), 145.4 (Ar-*C*) ppm.

5.4.2. Synthesis of 5,6-Dibromo-1,1,3,3-tetramethylisoindoline 336



Hydrogen peroxide (998 µL, 9.7 mmol, 30% in H_2O, 2.00 equiv.) was added dropwise to a suspension of 2,5,6-tribromo-1,1,3,3-tetramethylisoindoline **335** (2 g, 4.85 mmol, 1 equiv.) and $NaHCO_3$ (612 mg, 7.28 mmol, 1.5 equiv.) in MeOH/DCM (10:2 mL) and the resulting reaction mixture was stirred for 10 min and concentrated *in vacuo*. The residue obtained was taken up in Et_2O (150 mL), washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give 5,6-dibromo-1,1,3,3-tetramethylisoindoline **336** as a beige solid (1.61 g, 100%). Mp. 76–77 ^{o}C (Lit.,[50] 76-77°C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.41 (s, 12 H, C*H*$_3$), 1.94 (s, 1 H, N*H*), 7.34 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 33.7 (C-*C*H_3), 62.6 (*C*-CH_3), 122.9 (Ar-*C*), 126.8 (Ar-*C*), 150.2 (Ar-*C*) ppm.

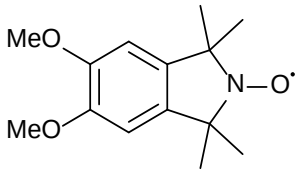
5.4.3. Synthesis of 5,6-Dimethoxy-1,1,3,3-tetramethyl isoindoline 337



A reaction mixture containing 5,6-bromo-1,1,3,3-tetramethylisoindoline **336** (3 g, 9 mmol, 1 equiv.), CuI (206 mg, 1.1 mmol, 0.12 equiv.), DMF (2 mL) and NaOMe (10 mL, 25% wt./v in MeOH) was refluxed for 24 h under an Ar atmosphere. The reaction was allowed to cool to RT, then diluted with water (15 mL) and extracted with Et_2O (70 mL x 4). The combined Et_2O extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give 5,6-dimethoxy-1,1,3,3-tetramethylisoindoline **337** as a white solid (1.86 g, 88%). The product was of sufficient purity and was used in subsequent steps without further

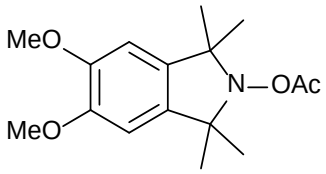
purification. Mp. 71-72 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.44 (s, 12 H, CH_3), 3.89 (s, 6 H, OCH_3), 6.60 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 32.0 (C-*C*H_3), 56.1 (O*C*H_3), 62.8 (*C*-CH_3), 104.4 (Ar-*C*), 140.3 (Ar-*C*), 148.8 (Ar-*C*) ppm.

5.4.4. Synthesis of 5,6-Dimethoxy-1,1,3,3-tetramethyl isoindolin-2-yloxyl 330



m-Chloroperoxybenzoic acid (1.85 g, 6.37 mmol, 1.5 equiv, 77%) was added to a solution of 5,6-dibromo-1,1,3,3-tetramethylisoindoline **337** (1 g, 4.25 mmol, 1 equiv.) in DCM (200 mL) at 0 °C. The cooling bath was removed after 30 min and stirring continued then at RT for a further 1.5 h. The resulting solution was washed with dilute HCl (0.5 M, 40 mL), NaOH (2 M, 40 mL x 2) and brine solutions and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude residue was purified by flash column chromatography (Hexane/EtOAc, 5:1) to give 5,6-dimethoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **330** as a bright yellow solid (925 mg, 87%). Mp. 202-203 °C (Lit.,[51] 201-203 °C).

5.4.5. Synthesis of 2-Acetoxy-5,6-dimethoxy-1,1,3,3-tetramethylisoindoline 333



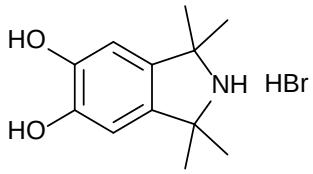
A reaction mixture containing 5,6-dimethoxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **330** (500 mg, 2 mmol, 1 equiv.) and Pd/C (50 mg, 10% wt.) in THF (15 mL) was flushed with Ar for 10 min. Then, a balloon of H_2 was connected and the reaction mixture was stirred for 20 min. The mixture was cooled in ice/H_2O bath followed by dropwise additions of TEA (558 µL, 4 mmol, 2 equiv.) and AcCl (284 µL, 4 mmol, 2 equiv.). The cooling bath was removed after 30 min and stirring was continued for a further 1.5 h. The reaction mixture was filtered through Celite and concentrated *in*

vacuo. The crude residue was dissolved in EtOAc (150 mL) and washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification of the crude product by silica gel column chromatography (Hexane/EtOAc, 5:1) gave 2-acetoxy-5,6-dimethoxy-1,1,3,3-tetramethylisoindoline **333** as a clear solid (557 mg, 95%). Mp. 84-85 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.38 (s, 6 H, CH_3), 1.46 (s, 6 H, CH_3), 2.20 (s, 3 H, C=OCH_3), 3.88 (s, 6 H, OCH_3), 6.61 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 19.3 (CO-*C*H_3) 25.4 (C-*C*H_3), 29.0 (C-*C*H_3), 56.2 (O*C*H_3), 68.3 (*C*-CH_3), 104.7 (Ar-*C*), 135.6 (Ar-*C*), 149.3 (Ar-*C*), 171.8 (*C*=OCH_3) ppm. HRMS (ES): *m*/*z* (%) = 294.1659 (100) [M + H]$^+$, calcd. for $C_{16}H_{23}NO_4$: 293.1625; found 293.1627. ATR-FTIR: υ_{max} = 2973 (m, ArC-H), 1753 (s, C=O), 1204 (s, C-N), 1170 (s, C-O) cm^{-1}.

5.4.6. Attempted Boron Tribromide Demethylation of 2-Acetoxy-5,6-dimethoxy-1,1,3,3-tetramethylisoindoline 333

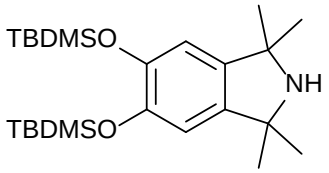
BBr_3 (2.56 mL, 2.56 mmol, 1 M solution in DCM, 3 equiv.) was added dropwise to a solution of 2-acetoxy-5,6-dimethoxy-1,1,3,3-tetramethylisoindoline **333** (250 mg, 852 μmol, 1 equiv.) in DCM (10 mL) at -78 ^oC under Ar. The resulting reaction mixture was allowed to return to RT. TLC analysis of the reaction mixture showed complete consumption of the starting material after 22 h. The excess BBr_3 reagent was quenched by careful addition of ice cold H_2O. The crude product was extracted with DCM (60 mL x 4) and the combined DCM extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. ^1H NMR analysis of the crude residue obtained showed complete demethylation. Attempts at purifying the crude mixture by column chromatography were unsuccessful.

5.4.7. Synthesis of 5,6-Dihydroxy-1,1,3,3-tetramethylisoindoline hydrobromide 154



A solution of 5,6-dimethoxy-1,1,3,3-tetramethylisoindoline **337** (500 mg, 2.12 mmol, 1 equiv.) in HBr (10 mL, 45% wt./v in H_2O) was refluxed overnight and then allowed to cool to RT. The resulting precipitate was filtered, washed with Et_2O (10 mL x 3) and dried under high vacuum to give 5,6-dihydroxy-1,1,3,3-tetramethylisoindoline hydrobromide **154** as grey solid (514 mg, 84%). Mp. 331-333 ^oC (dec.). ^1H NMR ($(CD_3)_2SO$, 400 MHz): δ = 1.58 (s, 12 H, CH_3), 6.63 (s, 2 H, Ar-*H*), 9.11 (s, 2 H, NH_2), 9.23 (s, 2 H, O*H*) ppm. ^{13}C NMR ($(CD_3)_2SO$, 100 MHz): δ = 29.1 (C-*C*H_3), 67.7 (*C*-CH_3), 108.4 (Ar-*C*), 132.9 (Ar-*C*), 147.0 (Ar-*C*) ppm. HRMS (ES): *m*/*z* (%) = 208.1308 (100) $[M]^+$, calcd. for $C_{12}H_{18}NO_2^+$: 208.1332; found 208.1308. ATR-FTIR: υ_{max} = 3147 (s, br, OH), 3008 (m, ArC-H), 1347 (s, C-N), 1237 (s, C-O) cm^{-1}.

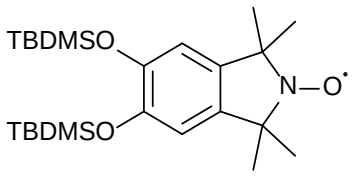
5.4.8. Synthesis of 5,6-Bis((tert-butyldimethylsilyl)oxy)-1,1,3,3-tetramethylisoindoline 340



A solution of 5,6-dihydroxy-1,1,3,3-tetramethylisoindoline hydrobromide **154** (100 mg, 347 µmol, 1 equiv.), TBDMSCl (261.5 mg, 1.74 mmol, 5 equiv.) and imidazole (118 mg, 1.74 mmol, 5 equiv.) in DMF (10 mL) was stirred under Ar for 4 days. The resulting reaction mixture was diluted with H_2O and basified to pH 10 with saturated $NaHCO_3$ solution. The aqueous layer was then extracted with Et_2O (60 mL x 4) and the combined Et_2O extracts were washed with dilute NaOH (0.5 M, 40 mL) and brine (30 mL x 5) solutions, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 5,6-bis((*tert*-butyldimethylsilyl)oxy)-1,1,3,3-tetramethylisoindoline **340** as a white solid (97 mg, 64%). The isolated product was of sufficient purity, as determined by ^1H NMR spectroscopy, and was used in the subsequent step without further purification. Mp. 105-107 ^oC. ^1H NMR ($CDCl_3$, 400 MHz): δ = 0.18 (s, 12 H, Si(CH_3)$_2$), 0.99 (s, 18 H, SiC(CH_3)$_3$), 1.38 (s, 12 H, CCH_3), 6.50 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 4.1 (Si(*C*H_3)$_2$), 18.4 (Si*C*$(CH_3)_2$, 26.0 (SiC(*C*H_3)$_3$), 31.8 (C-*C*H_3), 62.7 (*C*-CH_3), 113.6 (Ar-*C*), 141.0 (Ar-*C*), 146.1 (Ar-*C*) ppm. HRMS (ESI): *m*/*z* (%) = 436.3024 (25) $[M + H]^+$;

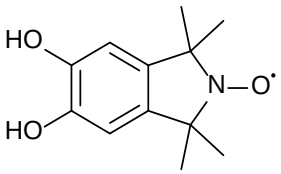
calcd. for $C_{24}H_{45}NO_2Si_2$: 435.2989; found 435.2987. FTIR (ATR): υ_{max} = 3100 (w, N-H), 2962 (m, ArC-H), 1233 (s, C-N), 1032 (s, C-O) cm^{-1}.

5.4.9. Synthesis of 5,6-Bis((tert-butyldimethylsilyl)oxy)-1,1,3,3-tetramethylisoindolin-2-yloxyl 341



m-Chloroperoxybenzoic acid (68 mg, 234 µmol, 1.2 equiv, 77%) was added to a reaction mixture containing 5,6-bis((*tert*-butyldimethylsilyl)oxy)-1,1,3,3-tetramethylisoindoline **340** (85 mg, 195 µmol, 1 equiv.) and $NaHCO_3$ (25 mg, 293 µmol, 1.5 equiv.) in DCM (15 mL) at 0 ^oC. The cooling bath was removed after 30 min and the stirring was continued RT for a further 1.5 h. The resulting solution was diluted (DCM, 100 mL), washed with dilute NaOH (0.5 M, 40 mL x 2) and brine (40 mL) solutions and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* to give 5,6-bis((*tert*-butyldimethylsilyl)oxy)-1,1,3,3-tetramethylisoindolin-2-yloxyl **341** as bright yellow solid (73 mg, 83%). Mp. 142-143 °C. HRMS (ESI): *m*/*z* (%) = 436.2821 (70) $[M + H]^+$, 450.2775 (100) $[M + Na]^+$; calcd. for $C_{24}H_4NO_3Si_2^{\bullet}$: 450.2860; found 436.2854._FTIR (ATR): υ_{max} = 2927 (m, ArC-H), 1343 (s, C-N), 1192 (s, C-O) cm^{-1}.

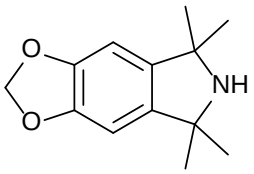
5.4.10. Synthesis of 5,6-Dihydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl 156



TBAF (2.44 mL, 2.44 mmol, 10 equiv., 1 M solution in THF) was added dropwise to a solution of 5,6-bis((*tert*-butyldimethylsilyl)oxy)-1,1,3,3-tetramethylisoindolin-2-yloxyl **341** (110 mg, 244 µmol, 1 equiv.) in THF (10 mL) and the resulting reaction mixture was stirred for 30 min at RT. The reaction mixture was concentrated under reduced pressure and the residue obtained was taken up in Et_2O (100 mL). The Et_2O

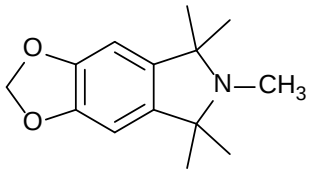
layer was washed with dilute HCl (0.5 M, 20 mL) and brine (40 mL), and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude product obtained was triturated with hexane to give 5,6-dihydroxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **156** as yellow solid (43 mg, 79%). Mp. 225-227 °C (dec.). HRMS (ESI): *m*/*z* (%) = 223.0926 (18) $[M + H]^+$; calcd. for $C_{12}H_{16}NO_3^{\bullet}$: 222.1130; found 222.1090. FTIR (ATR): υ_{max} = 3055 (s, br, O-H), 2961 (m, ArC-H), 1303 1234 (s, C-O) cm^{-1}.

5.4.11. Synthesis of 6,7-Dihydro-5H-[1,3]dioxolo[4,5-f]-5,5,7,7-tetramethylisoindoline (Methylenedioxy TMI) 155



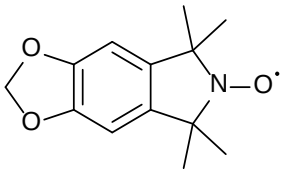
A solution of 5,6-dihydroxy-1,1,3,3-tetramethylisoindoline hydrobromide **154** (200 mg, 694 µmol, 1 equiv.), DCM (111 µL, 1.74 mmol, 2.5 equiv.) and NaOH (139 mg, 3.47 mmol, 5 equiv.) in DMSO was refluxed for 2 h and the resulting reaction mixture was allowed to cool to RT and then diluted with H_2O (50 mL). The crude product was extracted with DCM (60 mL x 4) and the combined DCM extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (DCM/5%MeOH) to give methylenedioxy TMI **155** as a white solid (108 mg, 71%). Mp. 125.5-126.5 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 1.49 (s, 12 H, C*H*$_3$), 5.96 (s, 2 H, OC*H*$_2$), 6.55 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 32.0 (C-*C*H_3), 62.6 (*C*-CH_3), 101.2 (Ar-*C*), 101.9 (*C*H_2), 141.7 (Ar-*C*), 147.2 (Ar-*C*) ppm. HRMS (ESI): *m*/*z* (%) = 220.1312 (100) $[M + H]^+$; calcd. for $C_{13}H_{17}NO_2$: 219.2840; found 219.2838. FTIR (ATR): υ_{max} = 3327 (m, br, O-H), 2962 (m, ArC-H), 1233 (s, C-N), 1032 (s, C-O) cm^{-1}.

5.4.12. Synthesis of 6,7-Dihydro-5H-[1,3]dioxolo[4,5-f]-5,5,6,7,7-pentamethylisoindoline (N-Methyl methylenedioxy TMI) 342



A solution of 5,6-dihydroxy-1,1,3,3-tetramethylisoindoline hydrobromide **154** (200 mg, 694 µmol, 1 equiv.), dibromomethane (97 µL, 1.39 mmol, 2 equiv.) and NaOH (139 mg, 3.47 mmol, 5 equiv.) in DMSO was refluxed for 2 h and the resulting reaction mixture was allowed to cool to RT and then diluted with H_2O (40 mL). The crude product was extracted with DCM (60 mL x 4) and the combined DCM extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (DCM) to give *N*-methyl methylenedioxy TMI **342** as low melting beige solid (118 mg, 73%). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.28 (s, 12 H, C*H*$_3$), 2.39 (s, 3 H, NC*H*$_3$), 5.93 (s, 2 H, OC*H*$_2$), 6.59 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 26.9 (C-*C*H$_3$), 27.1 (N-*C*H$_3$), 64.3 (*C*-CH$_3$), 101.0 (O*C*H$_2$), 102.1 (Ar-*C*), 140.8 (Ar-*C*), 146.8 (Ar-*C*) ppm. HRMS (ESI): *m*/*z* (%) = 234.1451 (18) $[M + H]^+$; calcd. for $C_{14}H_{19}NO_2$: 233.1416; found 233.1418. FTIR (ATR): υ_{max} = 2960 (m, ArC-H), 1304 (s, C-N), 1038 (s, C-O) cm^{-1}.

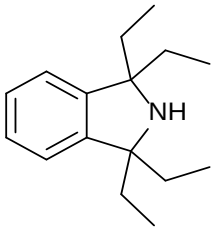
5.4.13. Synthesis of 6,7-Dihydro-5H-[1,3]dioxolo[4,5-f]-5,5,6,7,7-pentamethylisoindolin-6-yloxyl (Methylenedioxy TMIO) 157



m-Chloroperoxybenzoic acid (258 mg, 889 µmol, 1.3 equiv, 77%) was added to a reaction mixture containing methylenedioxy TMI **154** (150 mg, 684 µmol, 1 equiv.) and $NaHCO_3$ (86 mg, 1 mmol, 1.5 equiv.) in DCM (100 mL) at 0 ^{o}C. The cooling bath was removed after 30 min and the mixture was stirred at RT for a further 1.5 h. The resulting solution was washed with dilute NaOH (2 M, 20 mL x 2) and brine and

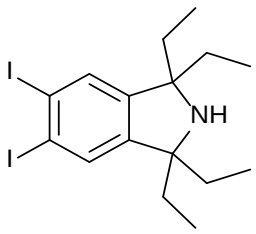
then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude residue was purified by flash column chromatography (Hexane/EtOAc, 5:1) to give methylenedioxy TMIO **157** as a bright yellow solid (143 mg, 89%). Mp. 188-189 °C. HPLC purity (>95%). HRMS (ESI): *m/z* (%) = 236.1612 (55) $[M + 2H]^+$, 257.0991 (100) $[M + Na]^+$; calcd. for $C_{13}H_{16}NO_3^{\bullet}$: 234.1130; found 234.1132. FTIR (ATR): υ_{max} = 2978 (m, ArC-H), 1303 (s, C-N), 1036 (s, C-O) cm^{-1}.

5.4.14. Synthesis of 1,1,3,3-Tetraethylisoindoline 344



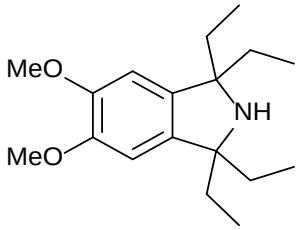
A suspension of 2-benzyl-1,1,3,3-tetraethylisoindoline **343** (2 g, 6.22 mmol, 1 equiv.) and Pd/C (200 mg, 10% wt.) in AcOH (30 mL) was shaken under an atmosphere of H_2 (50 psi in a Parr apparatus) for 3 h. The resulting mixture was filtered through Celite, concentrated under reduced pressure and basified with NaOH (1 M). The residue was extracted with DCM (60 mL x 4) and the DCM layer was washed with brine (40 mL). The DCM layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 1,1,3,3-tetraethylisoindoline **344** as pale yellow oil (1.35 g, 94%). The product was of sufficient purity by ^1H NMR spectroscopy and was used in the subsequent step without further purification. ^1H NMR ($CDCl_3$, 400 MHz): δ = 0.88 (t, 12 H, *J* = 7.2 Hz, C*H*$_3$), 1.70 (m, 8 H, *J* = 7.2 Hz, C*H*$_2$), 7.07 (m, 2 H, Ar-*H*), 7.21 (m, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 8.9 (*C*H_3), 33.8 (*C*H_2), 68.3 (*C*-CH_2), 122.4 (Ar-*C*), 126.5 (Ar-*C*), 147.5 (Ar-*C*) ppm.344

5.4.15. Synthesis of 5,6-Diiodo-1,1,3,3-tetraethylisoindoline 345



A solution of H_5IO_6 (969 mg, 3.49 mmol, 0.8 equiv.) in H_2SO_4 (15 mL, 98%) was cooled to 0 ^oC. KI (1.95 g, 11.77 mmol, 2.7 equiv.) was then added portionwise and the resulting dark-brown iodinium solution was stirred for 20 min. The iodinium solution was then added dropwise to a solution of 1,1,3,3-tetraethylisoindoline **344** (1.2 g, 4.36 mmol, 1 equiv.) in H_2SO_4 (10 mL, 98%). The resulting mixture was stirred for 3 h and then poured onto ice (200 mL) with care. The solution was basified with NaOH (10 M) and extracted with DCM (60 mL x 4). The combined DCM extracts were washed with saturated $Na_2S_2O_3$ (30 mL x 2) and brine (40 mL) solutions, and then dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the crude residue obtained was purified by silica gel chromatography (DCM) to give 5,6-diiodo-1,1,3,3-tetraethylisoindoline **345** as an off-white solid (1.68 g, 80%). Mp. 80–81 °C (Lit.,[49] 80–81 °C). ^1H NMR ($CDCl_3$, 400 MHz): δ = 0.85 (t, 12 H, *J* = 7.6 Hz, C*H*$_3$), 1.60 (m, 8 H, *J* = 7.6 Hz, C*H*$_2$), 7.55 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 8.8 (*C*H_3), 33.5 (*C*H_2), 68.1 (*C*CH_2), 105.1 (Ar-*C*), 133.4 (Ar-*C*), 150.3 (Ar-*C*) ppm.

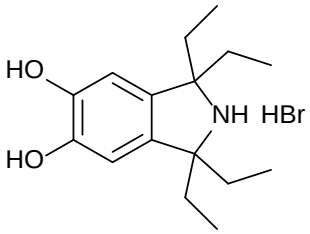
5.4.16. Synthesis of 5,6-Dimethoxy-1,1,3,3-tetraethylisoindoline 346



A reaction mixture containing 5,6-iodo-1,1,3,3-tetramethylisoindoline **345** (2.5 g, 5.17 mmol, 1 equiv.), CuI (118 mg, 620 μmol, 0.12 equiv.), DMF (5 mL) and NaOMe (15 mL, 25% wt./v in MeOH) were refluxed for 24 h under an Ar atmosphere. The reaction was allowed to cool to room temperature, then diluted with water and extracted with Et_2O (60 mL x 4). The combined Et_2O extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography ($CHCl_3$) to give 5,6-dimethoxy-1,1,3,3-tetraethyl isoindoline **346** as a white solid (1.236 g, 82%). Mp. 58-59 ^oC. HPLC purity (>95%). ^1H NMR ($CDCl_3$, 400 MHz): δ = 0.87 (s, 12 H, *J* = 7.6 Hz, C*H*$_3$), 1.67 (m, 8 H, *J* = 7.6 Hz, C*H*$_2$), 3.87 (s, 6 H, OC*H*$_3$),

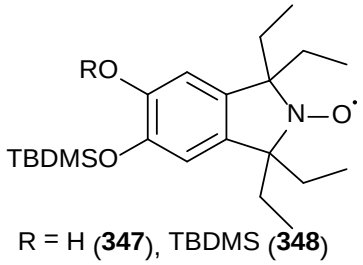
6.55 (s, 2 H, Ar-*H*) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 9.0 (*C*H_3), 33.9 (*C*H_2), 56.1 (O*C*H_3), 68.3 (*C*-CH_2), 120.3 (Ar-*C*), 105.5 (Ar-*C*), 139.2 (Ar-*C*), 148.3 (Ar-*C*) ppm. FTIR (ATR): υ_{max} = 3380 (w, N-H), 2960 (m, ArC-H), 1218 (s, C-N), 1162 (s, C-O) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 192.2229 (100) $[M + H]^+$; calcd. for $C_{18}H_{29}NO_2$: 191.2198; found 191.2196.

5.4.17. Synthesis of 5,6-Dihydroxy-1,1,3,3-tetraethylisoindoline 158 (Catecholamine TEI)



A solution of 5,6-dimethoxy-1,1,3,3-tetraethylisoindoline **346** (250 mg, 858 µmol, 1 equiv.) in aqueous HBr (5 mL, 48%) was refluxed overnight and the reaction mixture allowed to return to RT. The resulting precipitate was filtered, washed with Et_2O (10 mL x 3) and dried under high vacuum to give a grey powder of 5,6-dihydroxy-1,1,3,3-tetraethylisoindoline **158** as the hydrobromide salt (243 mg, 83%). Mp. 251-252 °C (dec.). ^{1}H NMR ($(CD_3)_2SO$, 400 MHz): δ = 0.92 (t, 12 H, *J* = 8 Hz, C*H*$_3$), 1.91 (m, 8 H, *J* = 8 Hz, C*H*$_2$), 6.6 (s, 2 H, Ar-*H*), 8.48 (s, 2 H, N*H*$_2$), 9.23 (s, 2 H, O*H*) ppm. ^{13}C NMR ($(CD_3)_2SO$, 100 MHz): δ = 8.7 (*C*H_3), 30.1 (C*H*$_2$), 74.6 (*C*-CH_2), 110.2 (Ar-*C*), 131.4 (Ar-*C*), 146.5 (Ar-*C*) ppm. HRMS (ES): *m*/*z* (%) = 264.1955 (100) $[M]^+$, calcd. for $C_{16}H_{26}NO_2^+$: 264.1958; found 264.1955. ATR-FTIR: υ_{max} = 3343 (s, br, OH), 2968 (m, Ar*CH*), 1303 (s, C-N), 1174 (s, C-O) cm^{-1}.

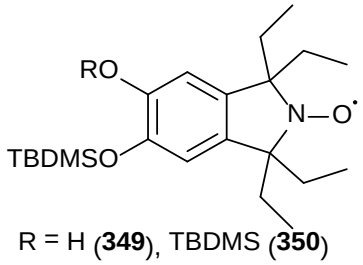
5.4.18. Synthesis of 5-Hydroxy-6-((tert-butyldimethylsilyl)oxy)-1,1,3,3-tetraethylisoindoline 347 and 5,6-Bis((tert-butyldimethylsilyl)oxy)-1,1,3,3-tetraethylisoindoline 348



R = H (**347**), TBDMS (**348**)

A solution of 5,6-dihydroxy-1,1,3,3-tetraethylisoindoline hydrobromide **158** (100 mg, 290 µmol, 1 equiv.), TBDMSCl (219 mg, 1.45 mmol, 5 equiv.), imidazole (99 mg, 1.45 mmol, 5 equiv.) in DMF (10 mL) was stirred under Ar for 4 days. The resulting reaction mixture was diluted with H_2O and basified to pH 9 with saturated $NaHCO_3$ solution. The aqueous layer was then extracted with Et_2O (60 mL x 4) and the combined Et_2O extracts were washed with brine (30 mL x 5), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product isolated was a mixture of mono- and di- silyl ether protected compounds **347** and **348** (123 mg) as verified by ^{1}H NMR spectroscopy and HRMS. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = [mono silyl ether **347** peaks] 0.1 (s, Si(C*H*$_3$)$_2$), 0.85 (t, CH$_2$C*H*$_3$), 0.91 (s, SiC(C*H*$_3$)$_3$), 1.6 (m, C*H*$_2$CH$_3$), 6.47 (s, 2 H, Ar-*H*) ppm; [di silyl ether **348** peaks] 0.096 (s, Si(C*H*$_3$)$_2$), 0.85 (t, CH$_2$C*H*$_3$), 0.91 (s, SiC(C*H*$_3$)$_3$), 1.62 (m, C*H*$_2$CH$_3$), 6.56 (s, 2 H, Ar-*H*) ppm. HRMS (ESI): *m*/*z* (%) = [mono silyl ether **347**] 378.2746 (100) $[M + H]^+$; calcd. for $C_{22}H_{39}NO_2Si$: 377.2750; found 377.2750; [di silyl ether **348**] 492.3651 (100) $[M + H]^+$; calcd. for $C_{28}H_{53}NO_2Si_2$: 491.3615; found 491.3618. FTIR (ATR): υ_{max} = 3236 (m, br, O-H), 2929 (m, ArC-H), 1349 (s, C-N), 1249 (s, C-O) cm^{-1}.

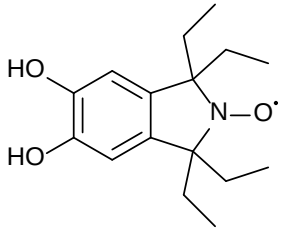
5.4.19. Synthesis of 5-Hydroxy-6-((tert-butyldimethylsilyl)oxy)-1,1,3,3-tetraethylisoindolin-2-yloxyl 349 and 5,6-Bis((tert-butyldimethylsilyl)oxy)-1,1,3,3-tetraethylisoindolin-2-yloxyl 350



R = H (**349**), TBDMS (**350**)

m-Chloroperoxybenzoic acid (110 mg, 377 µmol, 1.3, 77%) was added to a reaction mixture containing $NaHCO_3$ (37 mg, 435 µmol) and the crude silyl ether TEI (mixture of **347** and **348**, 123 mg) in DCM (50 mL) at 0 ^oC. The cooling bath was removed after 30 min and stirring was continued at RT for a further 1.5 h. The resulting solution was washed with dilute NaOH (0.5 M, 30 mL) and brine solutions and dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* to give the silyl ether TEIO mixture of **349** and **350** as a bright yellow solid (98 mg). HRMS (ESI): *m*/*z* (%) = [mono silyl ether **349**] 394.2587 (100) $[M + 2H]^+$; calcd. for $C_{22}H_{38}NO_{23}Si$: 392.2621; found 392.2619; [di silyl ether **350**] 529.3377 (70) $[M + Na]^+$; calcd. for $C_{28}H_{52}NO_3Si_2^{\bullet}$: 506.3486; found 506.3485. FTIR (ATR): υ_{max} = 3235 (m, br, O-H), 2969 (m, ArC-H), 1331 (s, C-N), 1250 (s, C-O) cm^{-1}.

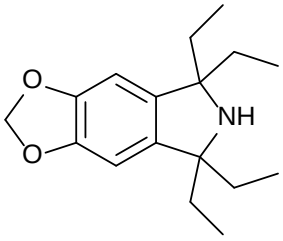
5.4.20. Synthesis of 5,6-Dihydroxy-1,1,3,3-tetraethylisoindolin-2-yloxyl 160 (catechol TEIO)



TBAF (2.9 mL, 2.9 mmol, 1 M solution in THF) was added dropwise to a solution of the silyl ether TEIO mixture (of **349** and **350**, 98 mg) in THF (10 mL) and stirred for 30 min at RT. The resulting reaction mixture was concentrated under reduced pressure and the residue obtained was dissolved Et_2O (60 mL x 4). The Et_2O layer

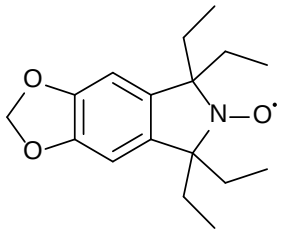
was washed with dilute HCl (0.5 M, 40 mL) and brine (40 mL) and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude product obtained was triturated with hexane to give 5,6-dihydroxy-1,1,3,3-tetraethylisoindolin-2-yloxyl **160** as a bright yellow solid (54 mg, 67% from **158**). Mp. 150-151 °C. HRMS (ESI): *m*/*z* (%) = 279.1792 (32) $[M + H]^+$, 301.1705 (40) $[M + Na]^+$; calcd. for $C_{16}H_{24}NO_3^{\bullet}$: 278.1756; found 278.1811. FTIR (ATR): υ_{max} = 3354 (s, br, O-H), 2972 (m, ArC-H), 1219 (s, C-O) cm^{-1}.

5.4.21. Synthesis of 6,7-Dihydro-5H-[1,3]dioxolo[4,5-f]-5,5,7,7-tetraethylisoindoline (Methylenedioxy TEI) 159



A solution of 5,6-dihydroxy-1,1,3,3-tetraethylisoindoline hydrobromide **158** (100 mg, 291 µmol, 1 equiv.), DCM (32 µL, 726 µmol, 2.5 equiv.) and NaOH (58 mg, 1.45 mmol, 5 equiv.) in DMSO (5 mL) was refluxed for 2 h and the resulting reaction mixture was allowed to cool to RT and then diluted with H_2O. The crude product was extracted with DCM (60 mL x 4) and the combined DCM extracts were washed with brine (40 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography ($CHCl_3$) to give methylenedioxy TEI **159** as a clear oil (low melting solid) (54 mg, 68%). HPLC purity (>95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.89 (t, 12 H, *J* = 7.6 Hz, C*H*$_3$), 1.67 (m, 8 H, *J* = 7.6 Hz, C*H*$_2$), 6.05 (s, 2 H, OC*H*$_2$), 6.59 (s, 2 H, Ar-*H*). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 8.9 (*C*H$_3$), 33.8 (C*H*$_2$), 68.2 (*C*-CH$_2$), 101.1 (Ar-*C*), 102.8 (*C*H$_2$), 140.3 (Ar-*C*), 146.7 (Ar-*C*) ppm. HRMS (ESI): *m*/*z* (%) = 276.1937 (100) $[M + H]^+$; calcd. for $C_{17}H_{25}NO_2$: 275.1885; found 275.1887. FTIR (ATR): υ_{max} = 3400 (w, N-H), 2950 (m, ArC-H), 1375 (s, C-N), 1041 (m, C-O) cm^{-1}.

5.4.22. Synthesis of 6,7-Dihydro-5H-[1,3]dioxolo[4,5-f]-5,5,7,7-tetraethylisoindolin-6-yloxyl (MethylenedioxyTEIO) 161



m-Chloroperoxybenzoic acid (68.5 mg, 236 µmol, 1.3 equiv., 77%) was added to a reaction mixture of methylenedioxy TEI **159** (50 mg, 182 µmol, 1 equiv.) and $NaHCO_3$ (23 mg, 273 µmol, 1.5 equiv.) in DCM (50 mL) at 0 ^oC. The resulting mixture was stirred for 30 min and then at RT for a further 1.5 h. The resulting solution was washed with dilute NaOH (2 M, 20 mL) and brine (30 mL) and then dried over anhydrous Na_2SO_4. The filtrate was concentrated *in vacuo* and the crude residue purified by flash column chromatography (Hexane/EtOAc, 6:1) to give methylenedioxy TEIO **161** as a bright yellow solid (45 mg, 85%). Mp. 75-76 °C. HPLC purity (>95%). FTIR (ATR): υ_{max} = 2968 (m, ArC-H), 1264 (s, C-N), 1037 (s, C-O) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 313.1648 (100) $[M + Na]^+$; calcd. for $C_{17}H_{24}NO_3^{\bullet}$: 290.1956; found 290.1749.

5.5 List of References

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Chapter 6: Conclusion and Future Work

6.1 Conclusion

As a wider strategy towards developing new therapeutics for managing chronic inflammation and oxidative stress-related diseases/disorders, the main aim of this PhD research project was to employ the pharmacophore hybridization strategy to design, synthesize and evaluate the therapeutic efficacy of novel potential dual-acting hybrid nitroxide-based antioxidants. The approach combines various nitroxide compounds with known pharmacophores - mainly the NSAID and the sympathomemitic stimulants class of drugs. The hybrid agents were constructed by either merging/overlapping the two structural subunits or via cleavable (ester and amide bonds) and non-cleavable (amine bond) linkers.

By taking advantage of the structural similarities of the parent templates, the novel overlapped nitroxide-salicylate hybrid agents (**137**, **138** and **139**) were designed and synthesized by merging the parent pyrroline nitroxide **3** with salicylic acid **135** and acetylsalicylic acid **136** templates. The cleavable and non-cleavable nitroxide-NSAID conjugates such as **140**, **141** and **143** were designed by linking various nitroxide compounds with selected NSAIDs (salicylic acid **135**, aspirin **136**, indomethacin **164** and 5-ASA **242**).

As anticipated, the merged analogues (**137** and **138**) displayed moderate antioxidant effect by inhibiting the MPO-mediated hypochlorous acid production system. Results from preliminary anti-inflammatory efficacy studies indicated that the merged hybrids (**137** and **138**) were less effective in the COX enzyme inhibitory assay than the parent aspirin **136**. Although the results are encouraging, notably, such COX inhibitory effects were only significant after longer incubation times. Thus, this result indicated that the merged nitroxide-salicylate derivatives are slow-time dependent COX inhibitors.

Studies to evaluate the antioxidant and anti-inflammatory effects of the novel nitroxide-NSAID conjugates (including **140**, **143**, **144**, **233** and **234**) were conducted

on the A549 Non-Small Cell Lung Cancer cells. In contrast to the merged counterparts, the nitroxide-NSAD conjugates displayed significant antioxidant and COX inhibitory effects similar to or in some instances better than the parent aspirin.

Notably, the nitroxide conjugate **140** showed a better ROS scavenging activity than the versatile antioxidant, TEMPOL. The ester-linked conjugates (**140**, **144**, **233** and **234**) possessed strong inhibitory effects on the COX enzyme than the amide-linked counterparts (**228**, **229** and **143**). Notably, the nitroxide conjugate **140** possessed better inhibitory effect on the COX enzyme than parent aspirin **136**. In addition to the antioxidant and anti-inflammatory effects, compound **140** was shown to inhibit the proliferation of A549 NSCLC cells. Clonogenic assay studies of A549 cells indicated that combining the conjugate **140** with anticancer agent gefitinib produced a synergistic effect on inhibiting A549 cells growth. The anticancer effect of compound **140** on A549 cell growth is quite promising and further studies are currently being undertaken.

In addition to the nitroxide-NSAID derivatives, this PhD project also explored the design and synthesis of novel merged nitroxide-sympathomemitic stimulants namely the nitroxide analogues of L-Dopa **147** and Ritalin **148**. Although the key and ultimate step (dirhodium-catalyzed intermolecular C-H insertion of methyl phenyldiazoacetate **309** into *N*-Boc-piperidine **306** to give the D-*threo*-methylephenedate TMIO methoxyamine derivative **317**) in the synthesis of the Ritalin-nitroxide analogue **150** was unsuccessful, the Schöllkopf's bis-lactim ether asymmetric synthesis of α-amino acids approach was successfully employed to synthesize the target L-Dopa nitroxide derivative **145**. By combining the Schöllkopf's methodology with the palladium-catalyzed Sonogashira coupling reaction, the rigid alkyne-linked chiral α-amino acid SDSL **151** was also synthesized. The synthesis of both the L-Dopa TMIO **149** and the alkyne-linked chiral α-amino acid SDSL **151** proceeded smoothly with high enantiomeric purity as verified by the ^{1}H NMR spectroscopy analysis of their respectively diastereomeric precursors **287** and **296**.

The dual-acting hybrid pharmacophore approach was also extended to catecholamine and benzodioxole-based compounds. In **Chapter 5**, the synthesis of a series of

isoindoline-based catecholamine and methylenedioxy compounds (**154-161**) was described. As the first class of catechol-based nitroxides, the target nitroxide-catecholinic-hybrids were mainly designed as potential dual-acting antioxidant therapeutics which could also be readily employed as synthetic templates/precursors for building functional materials. The target nitroxide-catecholinic-hybrids were designed to provide effective antioxidant intervention since the therapeutic benefits of common catechol and benzodioxole-based pharmacophores have been limited by the oxidative stress-induced cellular damaged linked to their long term use.

Some synthetic transformations carried out in this work that should be highlighted include

- Optimization of the copper-catalyzed methanolysis and cyanation of aryl halides.
- The use of the acetyl protecting group strategy under boron tribromide mediated demethylation conditions.
- Sodium aceloxyborohydride facilitated-chemoselective reduction of aryl amide to the corresponding amine in the presence of esters (employed in the synthesis of AAD-2004 TMIO analogue **142**).
- The Schöllkopf's asymmetric nucleophilic derivatization approach used in the synthesis of the novel chiral α-amino acids (**149** and **151**).
- The palladium-catalyzed Sonogashira coupling reaction conditions for the synthesis of the rigid alkyne-linked α-amino acid nitroxide (**151**).
- The palladium-catalyzed decarboxylative coupling of bromoamine precursor **23** and potassium malonate **316** in the synthesis of methyl ester **223**.
- The Regitz diazo transfer reaction employed in the synthesis of the diazo ester **309**.
- The dirhodium-catalyzed intermolecular C-H insertion reaction between *N*-Boc piperidine **306** and the transient metal carbene generated *in situ* from α-diazo methyl ester **309** in the attempted synthesis of Ritalin-nitroxide analogue **150**.
- The silyl ether protecting group approach employed in the synthesis of catechol nitroxides **156** and **160**.

6.2 Future work

The findings in this PhD project have certainly contributed to the use of the pharmacophore hybridization strategy as a practical and cost effective rational drug design and development approach. Since the results obtained from the design and development of the novel potential dual-acting nitroxide-based drug candidates were encouraging, it is therefore anticipated that this work could serve as a useful template for future drug design research that could further explore the antioxidant capacity of stable nitroxide compounds. Despite the successful synthesis of almost all of the target compounds (except Ritalin-TMIO analogue) in this work, only a few selected NSAID-nitroxide analogues were evaluated for their therapeutic efficacy. Therefore, the ongoing and future work in this project is centred mainly on evaluating the dual-acting therapeutic effects of the novel hybrid agents and/or their functional application. For instance, to fully determine the extent of the inhibitory effects of the merged nitroxide-NSAID hybrids (**137**-**139**) on the COX enzyme (and whether they are slow-time dependent COX inhibitors as was proposed), it is vital to run the COX inhibition experiments with these hybrids with incubation (over 20 min). Although the preliminary results obtained for the antioxidant and anti-inflammatory effects of the nitroxide-NSAID conjugates were conducted with only nine selected compounds, currently, further experiments are being undertaken (with all target nitroxide-NSAID conjugates) to give a thorough insight into their efficacies as well as the structure-activity relationships. In the case of the overlapped hybrid L-Dopa TMIO, further investigation should focus on evaluating its dual therapeutic efficacy: as an anti-Parkinson agent (potential dopamine replacement therapeutic) that could also provide remedy for oxidative stress damage, whether drug-induced or from the PD pathogenesis or both.

Experiments are currently planned through collaborations to incorporate and the rigid alkyne-linked chiral α-amino acid SDSL **151** into a peptide and subsequently study the structural and dynamic nature of the macromolecule through EPR spectroscopy and fluorescence.

Furthermore, it would be worthwhile to evaluate different dirhodium catalysts (such as Rh_2(S-biTISP)$_2$) for the intermolecular C-H insertion reaction between *N*-Boc

piperidine **306** and the transient metal carbene generated *in situ* from α-diazo methyl ester **309**.

Also, since the nitroxide-NSAID conjugate **140** possessed synergistic anti-cancer effects when combined with the anti-cancer agent gefitinib **350**, synthesizing and biological evaluation of a gefitinib-nitroxide conjugate such as **351** could be intriguing. **Scheme 6.1** below outlines the proposed route for the synthesis of the gefitinib-nitroxide conjugate **351**.

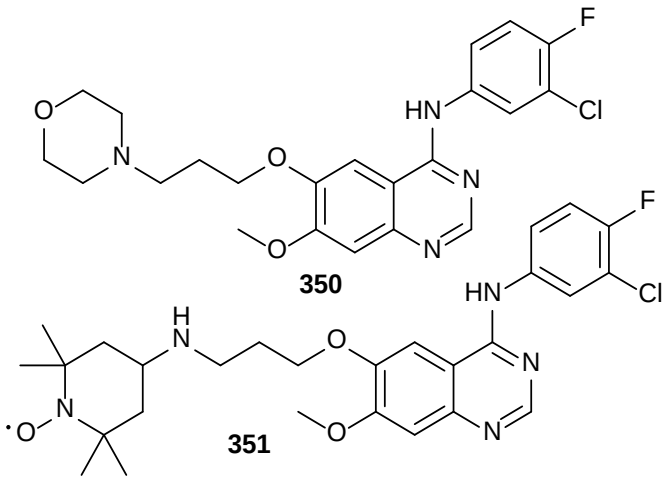
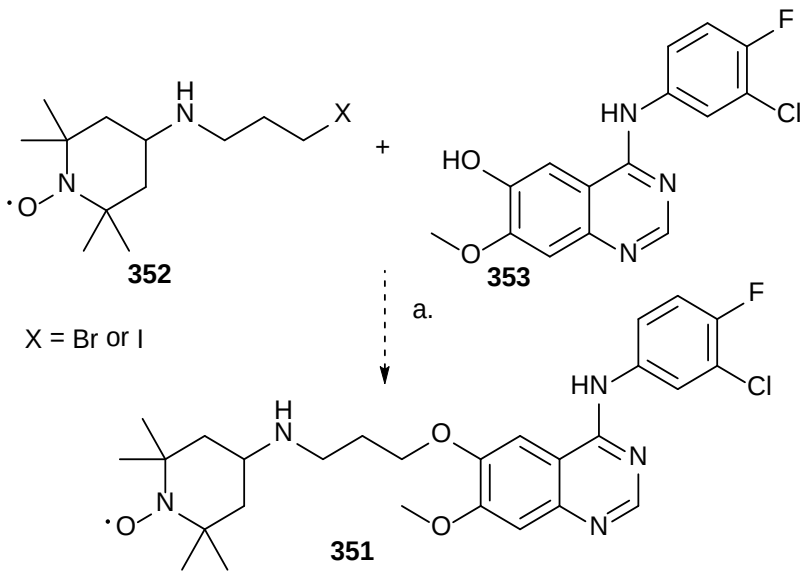


Figure 6.1. Chemical structure of gefitinib **350** and its amino-TEMPO analogue **351**.



Scheme 6.1. Proposed synthetic route to gefitinib amino-TEMPO derivative **351**. Reagents and conditions: a. K_2CO_3, DMF, reflux.